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Immunochemistry of O and R Antigens of *Salmonella* and Related *Enterobacteriaceae*

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The frequent overlapping reactions of *Salmonella* bacilli have not been the object of chemical investigation. One may expect that such studies will provide information on the apparent mosaic structure of antigens. Evidence for the existence of separate chemical entities underlying the serological reactions may be gained by demonstrating several specific groupings in homogeneous, well purified polysaccharides.

LANDSTEINER, 1945 (104)

INTRODUCTION

The genus *Salmonella* has been studied most intensively by bacteriologists and serologists. One indication of this great interest is the fact that today about 1,000 serotypes (or species) are known, and new additions to this list become available each year. The separation of one serotype from another is based on the different specificities of the thermolabile flagellar H antigens and the thermostable somatic O antigens.

The O antigen is a constitutive part of the bacterial cell wall (22, 68*b*, 100, 137, 193*a*, 261, 264), and is a complex composed of O-specific polysaccharide, lipid, and protein, which can be isolated by various extraction procedures. Besides possessing O-antigenic properties, this complex of high molecular weight exerts characteristic pharmacological activities in higher animals and man in doses of the order of 0.001 to 0.01 μg/kg. These include the production of fever, changes in the white blood cell count, enhancement of hormonal and enzymatic activities (for instance proteolysis), stimulation of phagocytosis and other defense mechanisms, etc. (17, 33, 236, 249). Higher doses than required for the above provoke the Shwartzman phenomenon (105, 236), tumor necrosis (20, 64, 84, 194, 198, 198*a*), and other toxic effects. In view of these properties, the complex is generally referred to as *endotoxin* (*see also* 157, 158, 159).

All *Enterobacteriaceae* and most gram-negative bacteria produce endotoxin. O-antigenic and endotoxic properties are closely associated, although the structures responsible for O specificity can be distinguished from those responsible for endotoxic activities (249, *see* 103, 123*b*).

White and Kauffmann may be credited with having used the serological relationships among different *Salmonella* to classify these organisms in terms of their O and H antigen specificities. This classification, known as the Kauffmann-White scheme, provides a theoretical as well as a practical basis for epidemiological and diagnostic studies. It also serves as a useful guide in comparative immunochemical, biochemical, and genetic investigations (88, 89).

When prepared by standardized procedures, antisera to *Salmonella*, often rendered highly specific by selective absorption, provide the reagents used to characterize the O antigens of individual strains. Classification of the different strains into some 40 serogroups has been achieved on the basis of the O-antigenic specificities.

Table 1 lists some representative strains according to the Kauffmann-White scheme. The members within each group show serological cross-reactions and are, therefore, considered to contain at least one common O factor, designated by a number. For instance, group B serotypes contain the common factor *4*; those of group D, factor *9*. Besides the common group factor, a given species may contain additional specificities (like factor *5*, or factor *19*), which occur exclusively in combination with a special group antigen (*4* and *3*, respectively). There are also several examples of inter-group cross-reactions. For instance, factor *12* is present in species of groups A, B, and D, and factor *1* appears in members of various groups (88, 89).

About 60 *different* O factors (specificities) have been differentiated thus far, and are used for classification in the Kauffmann-White scheme. The fact that several factors may occur in different combinations results in an even greater number of serotypes.

Finally, different relationships exist between O and H specificities, and the various combinations between these two classes of antigens (H and O) also serve to characterize the many serotypes.

Since the main purpose of the Kauffmann-White scheme is a practical (diagnostic) one, it has been kept as simple as possible. However, many specificities, represented in this scheme by one single number, have actually been shown to be multiple. For example, factor *12* consists of

TABLE 1. *Serological formulas and sugar composition of some Salmonella O antigens**

Serotype (species)	Group	O factors	Sugar constituents in addition to basal sugars†			Chemotype
S. paratyphi A	A	1, 2, 12	Man	Rha	Par	XV
S. paratyphi A var. *durazzo*		2, 12	Man	Rha	Par	
S. abortus equi	B	4, 12	Man	Rha	Abe	XIV
S. paratyphi B		1, 4, 5, 12	Man	Rha	Abe	
S. typhimurium		1, 4, 5, 12	Man	Rha	Abe	
S. typhimurium (mutant)		1, 4, 12	Man	Rha	Abe	
S. bredeney		1, 4, 12, 27	Man	Rha	Abe	
S. paratyphi C	C_1	6, 7	Man			III
S. cholerae suis		6, 7	Man			
S. newport	C_2	6, 8	Man	Rha	Abe	XIV
S. typhi	D	9, 12	Man	Rha	Tyv	XVI
S. sendai		1, 9, 12	Man	Rha	Tyv	
S. enteritidis		1, 9, 12	Man	Rha	Tyv	
S. anatum	E_1	3, 10	Man	Rha		XIII
S. newington	E_2	3, 15	Man	Rha		
S. illinois	E_3	(3), (15) 34	Man	Rha		
S. senftenberg	E_4	1, 3, 19	Man	Rha		
S. friedenau	G_1	13, 22	GalN	Fuc		VI
S. poona		13, 22	GalN	Fuc		
S. poona var. *37*		1, 13, 22, 37	GalN	Fuc		
S. worthington	G_2	1, 13, 23	GalN	Fuc		
S. minnesota	L	21	GalN			II
S. telaviv	M	28_1, 28_2	GalN	Rib		IX
S. dakar		28_1, 28_3	GalN	Rha		VIII
S. godesberg	N	30	GalN	Fuc		VI
S. adelaide	O	35	Col			X
S. inverness	P	38	GalN			II
S. riogrande	R	40	GalN	Man		IV
S. bukavu		1, 40	GalN	Man		
S. weslaco	T	42	Rha			VII
S. waycross		1, 42	Rha			
S. milwaukee	U	43	GalN	Fuc		VI
S. bergen	X	47	None			I

* From Kaufmann et al. (91). Selected according to the text of this review.

† Basal sugars: ketodeoxyoctonate, heptose, glucosamine, galactose, and glucose. Man = mannose; Rha = rhamnose; Fuc = fucose; GalN = galactosamine.

three distinct specificities *12_1*, *12_2*, and *12_3* (88). This is also true for factors which, according to the scheme, show only one group specificity; for example, factor *43* can be subdivided into four different specificities, all of which occur in group U but in different combinations (89, 96).

It has long been known that spontaneous mutations can and do occur in laboratory cultures. The mutants can be easily recognized, since they often form flat, rough (R) colonies, which contrast with the convex, smooth (S) colonies produced by the parent organisms (88, 99, 175). In liquid medium, most of the R forms agglutinate spontaneously and thus sediment, in contrast to the uniformly turbid cultures produced by the S forms. R-form mutants have lost their original

O specificity, but still carry the specific flagellar (H) antigen and generally exhibit the fermentation characteristics of the parent strains. In contrast to the numerous O specificities found in wild-type *Salmonella* strains, the mutants have acquired a new R specificity which, for a long time, was believed to be common to all R mutants, regardless of the smooth serotype from which they were derived. Later Kauffmann (88) showed that a limited number of R specificities could be distinguished in *Salmonella*, in *Escherichia coli*, and in *Shigella* R forms.

R mutants also produce endotoxins, which are closely associated with the R-antigenic complex (29, 30, 145, 171, 172, 221, 253). However, these strains are avirulent, and can be phagocytized much more easily than the wild strains (199, 201).

R mutants can readily be obtained from old cultures or cultures containing antisera against the S form, or with mutagenic substances. Many R mutants can be differentiated from the wild type by their altered reactivity to suitable phages, which are therefore of value in the selection and identification of R mutants (44, 167, 189, 190, 231).

As noted above, the classification of *Salmonella* in the Kauffmann-White scheme is based on differences in specificity of the somatic O antigens. Since the chemical structure of the determinant groups provides the molecular basis for the specificity, this review describes in some detail the investigations of the past 10 years that have related the immunological specificities of *Salmonella* O antigens to unique chemical structures.

From the chemical analyses of about 100 O antigens derived from different *Salmonella* serotypes, it became obvious that O antigens contain complex polysaccharides which may be composed of five to eight different monosaccharides (91). However, without exception, all *Salmonella* O antigens contain five common sugars: heptose, ketodeoxyoctonic acid, D-glucose, D-galactose, and D-glucosamine. The same sugars were found in polysaccharides derived from many R forms (93), no matter how complex in composition the the parent S forms. The working hypothesis was then developed that all-specific *Salmonella* polysaccharides might possess a common core made up of these sugars, to which are attached specific chains composed of the sugars characteristic of the serotype in question. These side chains manifest the O-antigenic specificity. Figure 1 shows schematically the two regions of an O polysaccharide: the common core and the specific side chains. It was found that the side chains are composed of repeating oligosaccharide units, symbolized by the rectangles in Fig. 1.

It was suggested that the common core could be composed of the polysaccharide present in R mutants (91, 93, 148). Actually, cross-reactions were obtained between certain S polysaccharides and R antisera. Moreover, such cross-reactions were obtained with other O polysaccharides also after partial degradation by weak acid hydrolysis (120).

Indeed, it became apparent that structural analyses of the R antigens would give information on the core, an aim difficult to attain with complete O antigens in which the core represents only a small part of the molecule.

The first part of this review deals with the immunochemical studies which provided an insight

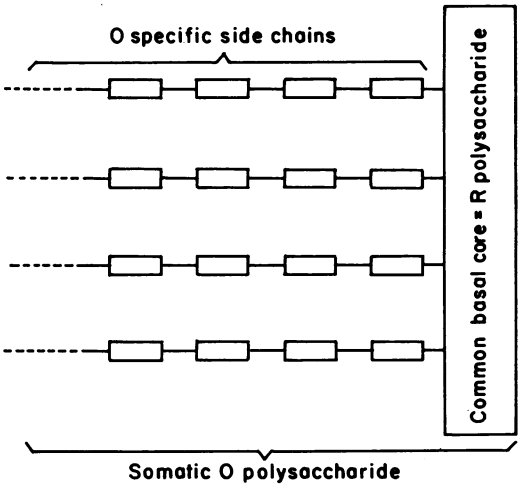


FIG. 1. *Schematic structural diagram of somatic Salmonella O polysaccharides.*

into the structural features of O-specific chains in a number of *Salmonella* groups, the chemical nature of some O factors carried by these chains, and the chemical modifications responsible for changes in specificity observed under the influence of phages. The second part is concerned with the chemical, biochemical, and genetic studies performed during the past few years on R mutants and their R antigens. The relation of R antigens to the basal core of O antigens will also be considered. The third part summarizes biosynthetic aspects, but only briefly, since they were reviewed recently by Osborn et al. (167).

A final section summarizes our present knowledge as well as unsolved problems in the chemistry, immunochemistry, and biochemistry of O antigens and their relation to R antigens. Biological properties of bacteria in relation to the composition of cell wall polysaccharides and their structure are also briefly discussed. The basis of

serological O specificity (O factors), as given by specific oligosaccharide units of the polysaccharide side chains, and the change of specificity by phage conversion resulting in defined changes of oligosaccharide structure are also discussed. This finally leads to considerations regarding approaches to a genetic classification of *Salmonella* and other *Enterobacteriaceae*.

STUDIES ON O ANTIGENS

Isolation and Properties of O-Specific Preparations from Bacteria

We owe much credit to Morgan, Goebel, and their co-workers for having done the principal pioneer work in this field. Morgan (140, 141) and Goebel (13, 54) showed that O antigens of gram-negative bacteria are complex macromolecules composed of polysaccharide, lipid, and protein, and that they may be isolated from whole bacteria or purified cell walls. Depending upon the goal of the investigation (*see* 159), various extraction procedures have been developed which lead to the isolation either of the complex whole antigen, or of its more or less degraded components. Only those methods will be mentioned here which have been used for preparations discussed in this review (Fig. 2). (For other methods, *see* 16, 27.)

Trichloroacetic acid extraction of the whole "Boivin" antigen. As originally proposed by Boivin and Mesrobeanu (14, 15), wet or acetone-dried bacteria are extracted with 0.25 N trichloroacetic acid at 4 C. The extracts are precipitated in the cold with 2 volumes of alcohol, taken up in a smaller amount of water, dialyzed, and lyophilized (81, 211). Such extracts are not homogeneous; their composition varies from species to species, and even (slightly) between preparations obtained from the same species. The major component is a complex antigen consisting of polysaccharide, lipid, and protein. It can be purified by gel filtration on a column of Sephadex (Skarnes, *personal communication*).

Injection of trichloroacetic acid extracts evokes the formation of agglutinins. They are also potent endotoxins.

Extraction of lipopolysaccharides with phenol-water, aqueous ether, and similar methods. A mixture of phenol and water (45:55) is used to extract lipopolysaccharide from bacteria at 68 C for 5 to 30 min (251, 259). After cooling, the upper aqueous phase is separated. It contains lipopolysaccharide, nucleic acid, and polysaccharide (for instance glucan), if the latter is present in the bacteria. Two extractions suffice to bring all of the lipopolysaccharide into aqueous solution. The dialyzed and concentrated extracts are centrifuged at high speed (100,000 × *g*). The pellet, after several washings, represents the lipopolysaccharide (42, 250, 259) consisting of a polysaccharide component, firmly bound lipid A, and small amounts of peptide. Lipopolysaccharide is a weak antigen in rabbits and in man (101, 265), but it exhibits O specificity and is a potent endotoxin (*see* 33, 103). Ribi and co-workers (46) introduced a method depending upon extraction with aqueous ether. This leads to a crude product, which, after several steps of purification including treatment with phenol-water, is distinct from the phenol-water product (251) in that the former contains a smaller quantity of firmly bound lipid (*see also* 159, 174*a*).

A variation of the phenol method consists of

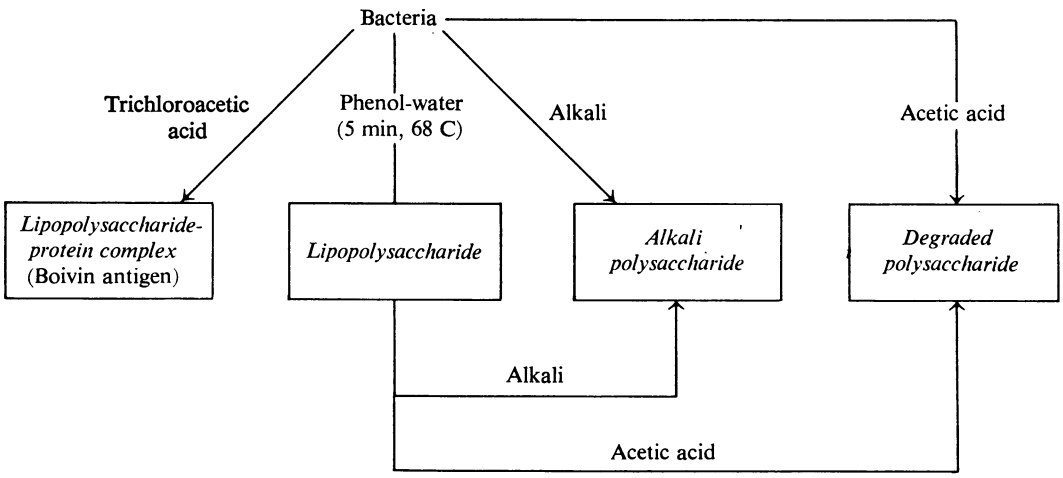


FIG. 2. *Preparation of different O-specific extracts from bacteria.*

pretreatment of the living or dried bacteria with formaldehyde before extraction with phenol-water, in which case the extracts do not contain nucleic acid (*unpublished data*). A similarly effective method in which trichloroacetic acid is used instead of formaldehyde has been described by O'Neill and Todd (163).

In addition to O-antigenic lipopolysaccharide, some enterobacterial strains, particularly *Escherichia*, form capsular antigens such as Vi and K. The Vi antigen (*see* 221) and many K antigens of *E. coli* (but not all; *see for example* 8) are acid polysaccharides. Mucoid strains, in addition, form a slime (M substance), termed colanic acid by Goebel et al. (52, 187), which is also an acid polysaccharide. By extraction with phenol-water, all of these polysaccharides, together with the nucleic acid, are obtained in the aqueous phase. Each of these polysaccharides may be separated with the aid of cetavlon (cetyl-trimethyl ammonium bromide) (5, 79, 193; *see also* 1, 206, 207), as shown by Jann et al. (77, 77*a*, 77*b*, 160*a*, 259).

Extraction of polysaccharides with sodium hydroxide: alkali polysaccharide. Alkaline extraction of gram-negative bacteria, first used by Krumwiede and Cooper (102), was refined by Furth and Landsteiner (47), who obtained a purer polysaccharide. Thomas and Mennie (235) showed that such preparations have a high affinity for cell surfaces. When incubated with erythrocytes, they are immediately absorbed and provide the sensitized erythrocytes used in passive hemagglutination tests (23, 146, 147*a*, 208, 235).

Purified preparations are obtained as follows (215). Dried bacteria are treated at 56 C with 0.25 N NaOH for 5 hr. The extract is neutralized and treated successively with trichloroacetic acid and 90% phenol. Different preparations from the same strain may vary with respect to precipitability in a given antiserum. Alkali polysaccharides contain polysaccharide and part or all of the firmly bound O-deacylated lipid A.

Similar preparations may be obtained from lipopolysaccharides by alkaline hydrolysis (115, 116, 147, 234), and are widely used for sensitization of erythrocytes in passive hemagglutination tests.

Extraction of polysaccharide by acetic acid: degraded Freeman polysaccharide. White (262) introduced this method, which involves successive treatments of the bacteria with 0.1 N acetic acid at 90 C. According to Freeman (41), the combined extracts are purified by several precipitations with alcohol and glacial acetic acid. The products finally obtained from *Salmonella* group B or D_1 bacteria are pure polysaccharides, low in N (0.2 to 0.4%) and P (0.4 to 0.9%) (81, 212). An important advantage of this method is that the specific polysaccharide is extracted quantitatively from the cells (186). Furthermore, different preparations give reproducible results in serological tests; i.e., the same amount of polysaccharide precipitates the same quantity of protein from a given antiserum. This has been utilized for the titration of O polysaccharide in *S. typhi* cultures (213, 214, 222).

A modification of the Freeman method, which avoids the many precipitation steps, consists in applying the phenol-water method directly to the crude acetic acid extracts (59). In general, successful separation from other polysaccharides and from nucleic acid is dependent on the nature of the specific polysaccharide and its composition.

The specific polysaccharide is not antigenic in rabbits. It may be contaminated with other polysaccharides, such as glucans, if these are present in the cells.

Physical and biological properties of O-specific preparations. Boivin antigen and lipopolysaccharides are of relatively large particle size, of the order of 1 to 20 millions (82, 192, 194). They form opalescent solutions which may be clarified by the addition of small amounts of pyridine or deoxycholate (Ribi, *personal communication*). Also, treatment of lipopolysaccharides with alkali results in rapid degradation (disaggregation) to a molecular weight of 200,000 with the simultaneous liberation of fatty acids (147). It has also been observed that short incubation of lipopolysaccharides with serum results in enhancement of diffusion in agar-gel, indicating disaggregation of the molecule (19, 185, 197, 198, 198*a*). The same result was obtained recently with sodium dodecyl sulfate (11*a*). It may be that the true molecular weight of lipopolysaccharides approximates 200,000, as represented by the type of molecule produced by alkali treatment of the complex lipopolysaccharide. The larger particle size of lipopolysaccharides, in the order of several millions, is probably due to aggregation as a result of van der Waals attraction of lipidic groups (long-chain fatty acid esters). These are split off by alkali, whereas the backbone of lipid A [composed of glucosamine, phosphoric acid ester, and β-hydroxy myristic acid (18, 85, 156, 255)] remains bound to the polysaccharide. Mild acid treatment of bacteria, of lipopolysaccharide, or alkali-treated lipopolysaccharide leads to the release of lipid A (18, 41, 255) or its constituents and lipid-free polysaccharide, the so-called *degraded polysaccharide* with a molecular weight of the order of 20,000 to 30,000 (28). The molecular weight of lipid A was found to be of the order of 2,000 to 3,000 (18; Fromme, *unpublished data*). Lipopolysaccharides thus appear to have a

mosaiclike structure, comprising polysaccharide units with a molecular weight of approximately 20,000 to 30,000 and lipid A with a molecular weight of approximately 2,000 to 3,000.

Table 2 summarizes some of the biological properties of the different extracts. All show O specificity, but there may be qualitative or quantitative differences between the preparations. For instance, it is to be expected that treatment with alkali would split alkali-labile linkages originally present. This is true for *O*-acetyl groups which are present in some O antigens but not in their alkali-treated polysaccharide derivatives. If, as in the case of factors *5* and *10*, serological specificity is dependent upon the presence of *O*-acetyl antigenicity upon the degraded polysaccharide by chemical coupling to protein (55, 58, 59). While these two types of polysaccharides are neither toxic nor pyrogenic, the Boivin antigen and the lipopolysaccharide possess both of these properties (33, 236, 249, *see also* 103).

Sugar Constituents of the Specific Polysaccharides

With regard to the chemical composition and structure of the complex somatic O antigen of *Enterobacteriaceae*, most investigations thus far have been concerned with their respective polysaccharides, so that it is in this area that our knowledge is most advanced.

TABLE 2. *Some properties of different O-specific bacterial extracts*

Extract	Main components*	Antigenicity (rabbits)	Serological specificity	Capacity to fix complement	Endotoxic activity	Red blood cell sensitizing activity	Molecular wt
Boivin antigen	PS lipid A protein lipid	+++	O-specific	+	+++	+	Several millions
Lipopolysaccharide	PS lipid A	±	O-specific	+	+++	±	Several millions
Alkali-polysaccharide	PS deacylated lipid A	Only if fixed to red blood cells	O-specific, lacking alkali-labile O factors	+	±	+++	200,000
Degraded polysaccharide	PS	Only if coupled to protein	O-specific	−	−	−	20,000 to 30,000

* PS = polysaccharide.

groups (98, 241), the alkali polysaccharide will be devoid of these specificities. However, more general differences seem to exist between the preparations. This became apparent when quantitative precipitation curves were compared (215) or when gel precipitation (209, 197) or absorption of agglutinins from antisera was studied. In the latter test, only the Boivin antigen (trichloroacetic acid extract) could eliminate all agglutinins from an antibacterial rabbit serum (208, 209). This could not be obtained with either the lipopolysaccharides, the degraded polysaccharides, or the alkali polysaccharides, even when the latter were fixed to erythrocytes. The reason for this failure is not yet known.

Although degraded polysaccharides and alkali-treated lipopolysaccharides are devoid of immunogenicity in the rabbit, antibodies to the pure polysaccharides can be obtained by immunization with alkali polysaccharide fixed to stromata (210). It is also possible to confer

Most of the analyses have been performed with degraded (Freeman) polysaccharides or with lipopolysaccharides (phenol-water products). Earlier studies had already shown the complexity of the specific polysaccharides of a few *Salmonella* serotypes (26, 252) in terms of the multiple nature of their sugar components. Today, the antigens of at least one representative of each *Salmonella* group (see Table 1), of *E. coli* groups, and of other *Enterobacteriaceae* have been analyzed in several laboratories (27, 31, 91, 95, 186). In general, the results are in good agreement except for one or two sugars (xylose, hexuronic acid) (27, 31), whose occurrence in some O antigens has not been confirmed by other authors. Table 3 summarizes the sugar constituents found thus far in *Salmonella* and related O antigens. Besides hexosamines, hexoses, and 6-deoxyhexoses, which were previously shown to be widespread in natural polysaccharides and glycosides, various hitherto unknown sugars

were detected in enterobacterial cell wall polysaccharides.

6-Deoxyhexoses. Besides the commonly found L-rhamnose and L-fucose, 6-deoxy-L-talose has been detected in O antigens of *Enterobacteriaceae* (77) (see Table 3).

3,6-Dideoxyhexoses. In 1952–1953, the first representatives of this new class of chromatographically fast-moving deoxysugars were detected in hydrolysates of enterobacterial polysaccharides (168, 248, 254). Of the eight possible isomeric 3,6-dideoxyhexoses, five have thus far been found in cell wall polysaccharides of gram-negative bacteria (Table 4). In *Salmonella* O antigens, abequose, colitose, tyvelose, and paratose were found. In *Escherichia* O antigens, only colitose was detected, whereas the O antigens of *Pasteurella pseudotuberculosis* groups I to V (27) contained abequose, tyvelose, paratose, and ascarylose [originally discovered by Lederer and his co-workers (38) in *Ascaris* eggs]. 3,6-Dideoxyhexoses were also found in the genera of *Arizona* (colitose in *Arizona 9* and *20*) and *Citrobacter* [abequose in *Citrobacter* (*4*, *5*) (257)]. Two pairs of optical antipodes were recognized (260), i.e., abequose and colitose, tyvelose and ascarylose.

The structures of these sugars were established in collaboration by the groups of Lederer, Staub, and Westphal (37). The chemical properties, analyses, and syntheses, as well as the various

TABLE 3. *Monosaccharide constituents of antigens of Salmonella and related gram-negative bacteria*[a]

Hexosamines	Deoxyhexosamines	Hexoses	6-Deoxyhexoses	3,6-Dideoxyhexoses[b]
D-Galactosamine	L-Fucosamine[c]	*D-Galactose*	*L-Fucose*	*Abequose*
	D-Fucosamine[d]			*Colitose*
D-Glucosamine	D-Viosamine[e]	*D-Glucose*		*Paratose*
		D-Mannose	*L-Rhamnose*	*Tyvelose*
				Ascarylose
			6-Deoxy-L-talose	

2-Keto-3-deoxyoctonate	*L-Glycero-D-mannoheptose*[f]
Neuraminic acid	D-Glycero-D-galactoheptose[g]
Ribose, xylose[h]	D-Glycero-D-mannoheptose[i]

[a] Sugars found in *Salmonella* O antigens are italicized.
[b] See Table 4.
[c] *Enterobacteriaceae* (9).
[d] *Chromobacterium violaceum* (21).
[e] 4,6-Dideoxy-4-amino-D-glucose. *C. violaceum* (261, 226).
[f] (Or its optical antipode) *Shigella sonnei* phase II (78), *S. flexneri* (200), *Escherichia coli* B (246), *S. dysenteriae* (25, 27), *Salmonella* strains (3*a*, 98*a*, 165), *Proteus mirabilis* (3*a*), *Serratia marcescens* (1*a*).
[g] *C. violaceum* (124).
[h] *E. coli* O8 strain Kröger (114).
[i] *C. violaceum* (25, 27), *Proteus mirabilis* (3*a*, 98*a*), *Serratia marcescens* (1*a*).

TABLE 4. *Enterobacterial 3,6-dideoxyhexoses*

Trivial name	Chemical designation	
	Relation to other hexoses	International nomenclature
Abequose	3,6-Dideoxy-D-galactose (3-deoxy-D-fucose)	3,6-Dideoxy-D-*xylo*-hexose
Colitose	3,6-Dideoxy-L-galactose (3-deoxy-L-fucose)	3,6-Dideoxy-L-*xylo*-hexose
Tyvelose	3,6-Dideoxy-D-mannose (3-deoxy-D-rhamnose)	3,6-Dideoxy-D-*arabino*-hexose
Ascarylose	3,6-Dideoxy-L-mannose (3-deoxy-L-rhamnose)	3,6-Dideoxy-L-*arabino*-hexose
Paratose	3,6-Dideoxy-D-glucose	3,6-Dideoxy-D-*ribo*-hexose

biological aspects of these components, have been reviewed (256). Only a few points need be stressed here: O antigens may or may not contain a dideoxyhexose, but more than one member of this class has not yet been found in one antigen. The glycosidic linkage of these sugars is acid-labile. Since dideoxyhexoses, as far as we know, constitute nonreducing terminal end groups in the highly branched polysaccharides, they are the first to be released during hydrolysis. Glycosidically linked 3,6-dideoxyhexoses do not contain vicinal OH groups, and therefore are resistant to oxidation with periodate. Consequently, if a polysaccharide containing a dideoxyhexose is treated with periodate, this sugar is the only terminal unsubstituted monosaccharide which is not destroyed. Therefore, O specificity linked to the dideoxyhexoses is retained in periodate-treated polysaccharides. This proved to be important for the evaluation of the role played by 3,6-dideoxyhexoses in immunological specificity (216).

Heptoses. The first discovery of a heptose as constituent of O-antigenic lipopolysaccharides was made by Jesaitis and Goebel (78) in their studies on the somatic antigens of *Shigella sonnei*. Since then, heptoses were found in a large number of gram-negative O antigens. In fact, all strains of *Salmonella*, *Escherichia*, *Citrobacter*, *Arizona*, *Hafnia*, *Proteus*, and *Pasteurella* analyzed so far contain heptose in their lipopolysaccharide. We have found only one lipopolysaccharide with no heptose; this had been isolated from a *P. pestis* strain. *Salmonella* and *E. coli* lipopolysaccharides probably contain an identical heptose (77, 91). After short hydrolysis, the heptose is obtained esterified with phosphate. In these lipopolysaccharides, heptose is not substituted in C6 and C7, as oxidation with periodate of lipopolysaccharides not containing mannose, followed by reduction and hydrolysis according to Davies (27), results in the appearance of mannose (91, 164). Osborn (164) identified the heptose of a *S. typhimurium* mutant as L-glycero-D-mannoheptose. The same heptose was identified in *S. minnesota* mutants (3*a*). As can be seen from Table 3, two other heptoses were found, and still others may exist in O antigens of different gram-negative bacteria (see 27). Recently a *Proteus mirabilis* strain was shown to contain two heptoses in its lipopolysaccharide: the phosphoric ester of L-glycero-D-mannoheptose and D-glycero-D-mannoheptose (98*a*). Both these heptoses react with mannose isomerase to give the corresponding heptuloses. Their degradation according to Ruff leads to the corresponding hexoses (3*a*, *see also* 1*a*).

Hexosamines. Of the hexosamines in Table 3, D-glucosamine, like heptose, is a common constituent of *Salmonella* O-specific polysaccharides, though it may be present only in small amounts (2 to 4%). D-Galactosamine may also be present. L-Fucosamine has been found recently in some *Salmonella* species and in other *Enterobacteriaceae*, where it had escaped detection earlier (9, 9*a*). At present, it is not known whether or not this amino sugar is an integral part of the O antigen. The presence of D-fucosamine in *Chromobacterium violaceum* was described by Crumpton and Davies (21). (*See also* 185*a*.)

Okazaki and Strominger (162) isolated from *E. coli* R strains the thymidine diphosphate (TDP) derivatives of two amino sugars: TDP-4-acetamido-4,6-dideoxy-D-glucose (from *E. coli B*) and TDP-4-acetamido-4,6-dideoxy-D-galactose (from *E. coli K-12 Y10*) (226*a*, 226*b*). The enzymes making TDP-acetamido sugar from TDP-glucose (130) (see below, Fig. 10) were also found in *Salmonella* (including R strains from groups B, C_2, D, H, O, and T) and *Pasteurella* (*P. tuberculosis* types I to V). The sugar formed probably has the D-galactose configuration (Matsuhashi and Strominger, *personal communication*). In *S. minnesota* S and R strains, the enzyme making TDP-4-keto-6-deoxyglucose (see Fig. 10) from TDP-glucose was detected (123*a*), which is an intermediate in the synthesis of 4-amino-4,6-dideoxygalactose. The ability of many bacteria to synthesize this amino sugar suggests that it is a common constituent of O antigens of many *Enterobacteriaceae*, but that it escaped detection in hydrolysates because of its acid sensitivity. In some mutants in which the sugar is not transferred, the nucleotide may accumulate. 4-Amino-4,6-dideoxyglucose was isolated from *Chromobacterium violaceum* and termed viosamine (226).

Ribose. If detected in lipopolysaccharides, ribose mostly originates from contaminating ribonucleic acid. In some lipopolysaccharides, however, ribose or a ribose derivative is a true constituent, as in *Salmonella* antigens 28_1, 28_2; *52* and *56* (91, 95) (see T forms).

2-Keto-3-deoxy-octonate (*KDO*). KDO was isolated recently from *E. coli* O111 lipopolysaccharide by Heath and Ghalambor (66), who studied its structure and biosynthesis. Since then, KDO, as recognized by the periodate-thiobarbituric acid reaction and in some cases by paper chromatography and paper electrophoresis, has been found to be a common constituent of all O antigens and specific polysaccharides analyzed so far. Osborn suggested that KDO might serve as a

link between the polysaccharide and lipid A in lipopolysaccharides (see below).

O-phosphorylethanolamine. Recently, a new constituent of enterobacterial polysaccharides was discovered by Grollmann and Osborn (57), who isolated and identified O-phosphorylethanolamine in hydrolysates of lipopolysaccharides and polysaccharides derived from *Salmonella* and *E. coli*. It is believed to be an integral constituent of O antigens linked to heptose phosphate through phosphodiester linkages. Ikawa et al. (69) had already described the occurrence of ethanolamine in an *E. coli* lipopolysaccharide.

Sugar Composition of Specific Polysaccharides

More than 15 monosaccharides have thus far been found as constituents of *Salmonella* O antigens (see Table 3), of which from 5 to 8 build up each of the complex heteropolysaccharides. Table 1 summarizes the sugar analyses of some *Salmonella* polysaccharides.

According to their sugar composition, *Salmonella* O antigens have been classified into chemotypes (91, 95), each chemotype comprising antigens of the same qualitative sugar composition, from chemotype I (the simplest) to the most complicated chemotypes XIV-XVI (containing eight different sugars) (Table 5). The main finding of the qualitative chemical analyses was that the composition with respect to sugars of O antigens belonging to the same *Salmonella* serogroup was identical. A close correlation was thus found to exist between the classification of *Salmonella* serotypes into serogroups and the

TABLE 5. *Chemotypes of Salmonella (91, 95)**

Chemotype	Hexosamines: D-Galactosamine	Hexosamines: D-Glucosamine	KDO: 2-Keto-3-deoxy-octonate	Heptose: L-Glycero-D-manno-	Hexoses: D-Galactose	Hexoses: D-Glucose	Hexoses: D-Mannose	6-Deoxyhexoses: L-Fucose	6-Deoxyhexoses: L-Rhamnose	Pentoses: Ribose	3-6-Dideoxyhexoses: Colitose	3-6-Dideoxyhexoses: Abequose	3-6-Dideoxyhexoses: Paratose	3-6-Dideoxyhexoses: Tyvelose	O-Serogroups
I		●	●	●	●	●									J,V,X,Y,58
II	○	●	●	●	●	●									L,P,51,55
III		●	●	●	●	●	○								C_1,C_4,H,S
IV	○	●	●	●	●	●	○								K,R
V		●	●	●	●	●		○							W
VI	○	●	●	●	●	●		○							G,N,U
VII		●	●	●	●	●			○						T,59
VIII	○	●	●	●	●	●			○						M,(28_1,28_3)53,57
XXV		●	●	●	●	●				○					52
IX	○	●	●	●	●	●				○					M,(28_1,28_2)56
X		●	●	●	●	●					○				O
XI	○	●	●	●	●	●					○				Z
XII	○	●	●	●	●	●	○	○							I,Q
XIII		●	●	●	●	●	○		○						E,F,54
XIV		●	●	●	●	●	○		○			○			B,C2,C3
XV		●	●	●	●	●	○		○				○		A
XVI		●	●	●	●	●	○		○					○	D1,D2

* Shaded circles: sugars present in the basal core. Open circles: sugars present only in the specific side chains.

sugar composition of their respective O antigens (lipopolysaccharides) (91).

On the other hand, it may be seen from Table 5 that, in several classes, O antigens of strains from two serologically different groups may belong to the same chemotype. It is assumed that in these instances the same sugars are at least partly linked in different ways, which would explain the difference in specificity.

Similar investigations have been performed recently with more than 100 serologically classified *E. coli* strains, by I. and F. Ørskov and B. and K. Jann, who extended (77, 160*b*) preliminary studies of Kauffmann et al. (92). As can be seen from Table 6, 12 of the 16 *Salmonella* chemotypes were also identified in *E. coli* strains, whereas 4 higher *Salmonella* chemotypes were not found (IX, XIV, XV, XVI). On the other

TABLE 6. *Chemotypes of Escherichia coli*[a]

Chemotype	Hexosamines			KDO		Hexoses			6-Deoxy-hexoses				Serogroups[d]
	Galactosamine	2,6-Dideoxy-2-amino-hexose[b]	Glucosamine	2-Keto-3-deoxy-octonate[c]	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	6-Deoxy-talose	Colitose	
I			◐	◐	◐	◐	◐						**14, 24, 28, 30,** 32,38,42,56,82, 83,118,141
II	○		◐	◐	◐	◐	◐						21,22,23,27,33,37,46,61,81
III			◐	◐	◐	◐	◐	○					8,9,40.58,78,93
IV	○		◐	◐	◐	◐	◐	○					6
V			◐	◐	◐	◐	◐		○				41,52
VI	○		◐	◐	◐	◐	◐		○				86,127,128
VII			◐	◐	◐	◐	◐			○			**1,** 13,18,19,31,35,39,50,53,54, 60,99,100,102,119,129
VIII	○		◐	◐	◐	◐	◐			○			48,49,51
X			◐	◐	◐	◐	◐					○	111
XI	○		◐	◐	◐	◐	◐					○	55
XII	○		◐	◐	◐	◐	◐	○	○				11,43,125
XIII			◐	◐	◐	◐	◐	○		○			7,34,75
XVII			◐	◐	◐		◐	○					44,59,77
XVIII			◐	◐	◐	◐	◐	○	○				126
XIX			◐	◐	◐		●	○		○			17
XX		○	◐	◐	◐	◐	◐						15,57
XXI		○	◐	◐	◐	◐	◐			○			4,10,16,25,26
XXII		○	●	◐	◐	◐	◐				○		45
XXIII			◐	◐	◐	◐	◐						3
XXIV			◐	◐	◐	◐	◐	○	○	○			36

[a] After Kaufmann et al. (92), Jann (77), and Ørskov et al. (160*b*). The composition of *E. coli* O15, O25, and O26 differs from earlier analysis for sugars of strains of the same O groups (92). An additional 2-amino-6-deoxyhexose was found in their lipopolysaccharides (77). In *E. coli* O25, galactosamine was not found. In *E. coli* O73 galactose was found.

[b] The 2-amino-6-deoxyhexoses of the two columns are distinct.

[c] Tests for KDO were done with one or two members of each chemotype. It was always found to be present.

[d] Underlined figure represent *E. coli* serogroups showing cross-reaction with *Salmonella* serogroups.

hand, eight *E. coli* chemotypes (XVII to XXIV) have not yet been found to occur in *Salmonella* serotypes. There are some *E. coli* strains with lipopolysaccharides lacking galactose (chemotypes XVII and XIX).

Serological cross-reactions between genera may often be correlated with identical or similar composition of the respective antigens. This was demonstrated in many systems in which *Salmonella* serotypes cross-react with serotypes of *E. coli* or of *Arizona* or *Citrobacter*, or of both (Table 7) (257). However, these cross-reacting O

TABLE 7. *Sugar constituents of O antigens of various cross-reacting bacterial genera**

Organism	O Antigen	Galactosamine	Glucosamine	KDO	Heptose	Galactose	Glucose	Mannose	Fucose	Rhamnose	Colitose	Abequose	Tyvelose
Salmonella hvittingfoss	*16*	+	+	+	+	+	+	+	+				
Arizona	*25*	+	+	+	+	+	+	+	+				
Escherichia coli	*11*	+	+	+	+	+	+	+	+				
S. onderstepoort	(1), *6, 14, 25*		+	+	+	+	+	+					
E. coli	*73*		+	+	+	?	+	+					
S. weslaco	*42*		+	+	+	+	+			+			
Arizona	*15*		+	+	+	+	+			+			
E. coli	*31*		+	+	+	+	+			+			
S. inverness	*38*	+	+	+	+	+	+						
Arizona	*16*	+	+	+	+	+	+						
E. coli	*21*	+	+	+	+	+	+						
S. aberdeen	*11*		+	+	+	+	+	+		+			
Arizona	*17*		+	+	+	+	+	+		+			
E. coli	*75*		+	+	+	+	+	+		+			
S. adelaide	*35*		+	+	+	+	+				+		
Arizona	*20*		+	+	+	+	+				+		
E. coli	*111:B4*		+	+	+	+	+				+		
S. greenside	*50*	+	+	+	+	+	+				+		
Arizona	*9*		+	+	+	+	+				+		
E. coli	*55:B5*	+	+	+	+	+	+				+		
S. milwaukee	*43*	+	+	+	+	+	+		+				
Arizona	*21*	+	+	+	+	+	+		+				
E. coli	*86:B7*	+	+	+	+	+	+		+				
S. dakar	*28*	+	+	+	+	+	+			+			
Citrobacter	(*28*)	+	+	+	+	+	+			+			
S. djakarta†	*48*		+	+	+	+	+						
Citrobacter†	(*48*)		+	+	+	+	+						
S. paratyphi B	*4, 5, 12*		+	+	+	+	+			+		+	
Citrobacter	(*4, 5*)		+	+	+	+	+					+	
S. schleissheim	*4, 12, 27*		+	+	+	+	±	+		+		+	
P. pseudotuberculosis II	(*4, 27*)		+	+	+	+	+	±				+	
S. strasbourg	(*9*), *46*		+	+	+	+	±	+		+			+
P. pseudotuberculosis IV	[(*9*), *46*]	+	+	+	+		±	+					+

* From Kauffmann et al. (257) slightly modified according to Kauffmann et al. (95).
† Contains neuraminic acid in addition to the sugar constituents shown.

antigens need not be of the same chemotype, because serological cross-reactivity is related to analogous determinant structures which comprise only small parts of the whole antigen molecule (223).

Enterobacterial antigens frequently show serological cross-reactions with nonbacterial polysaccharides. For instance, Heidelberger and Cordoba (67, 68) found that yeast mannan was precipitated by horse antisera to *S. typhi*, and galactomannans were precipitated by horse antisera to *S. paratyphi B* and dextrans by both sera.

Many enterobacterial O antigens show cross-reactions with human blood group substances of the A, B, O system, especially those bacterial lipopolysaccharides which contain the same four sugar constituents as the human blood group substances (*see* 80, 245), namely *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-galactose, and L-fucose (35, 73, 74, 204, 205). For instance, the *E. coli* O86 antigen is a very potent blood group B antigen. The relationship between *Salmonella* factor *5* and Forssman and blood group A specificities (70, 136) can be attributed to the *O*-acetylgalactose responsible for the specificity of factor *5* (98). Indeed, 2-*O*-acetylgalactose would be immunologically very similar to *N*-acetylgalactosamine, which inhibits anti-*5* (98, 74), anti-A, and anti-Forssman (136, 74; *see* 245) sera. Serological relations found to exist between *Salmonella* O antigens of serogroup U and human blood group substance could also be related to analogous oligosaccharide structures (196). On the basis of serological relationships between many enterobacterial O antigens and blood group substances, Springer et al. (203) found that the isohemagglutinins are absent in germ-free animals but could be evoked by immunization or infection with cross-reacting enterobacterial strains.

Structure of the O-Specific Side Chains

As indicated by the classification into 17 chemotypes, *Salmonella* O antigens may be composed of different sugars. Without exception, however, they all contain the sugars of chemotype I, which are the five so-called "basal sugars": KDO, heptose, glucosamine, glucose, and galactose. O antigens which belong to the higher chemotypes contain additional sugars (Table 5).

These facts led to the concept that *Salmonella* polysaccharides might be composed of a common core containing the basal sugars, with long side chains attached to the core, as shown schematically in Fig. 1. The side chains of Fig. 1 may contain basal sugars, if the antigen belongs to chemotype I. They contain additional sugars, with or without the basal ones, if they belong to any other chemotype.

We shall now review the present knowledge of the structure of the specific chains.

Salmonella of group D_1. The first data on the structure of an O-specific polysaccharide were obtained with *S. typhi* (*9*, *12*). Glucose, galactose, mannose, rhamnose, and tyvelose, the main constituents of this polysaccharide, occur in about equimolar proportions (168–170). Heptose and glucosamine, constituents of the basal structure, are present only in small amounts.

Studies on its methylation were begun by Pon and Stacey (*unpublished data*) and were further developed by Tinelli (237). Hydrolysates of the fully methylated polysaccharide contained a small quantity of each of the sugars as a fully methylated derivative, indicating their terminal position in the polysaccharide. (The respective methylated tyvelose derivative is a very volatile substance, and was not isolated under the conditions used in this study.) It is not known whether these sugars were in terminal positions because of the method of preparation of the polysaccharide, or whether they are really linked terminally in the natural antigen. Also, dimethylated sugars were obtained. It was concluded that the polysaccharide was highly branched.

Oxidation of the polysaccharide by periodate left galactose, mannose, and tyvelose intact, whereas glucose and rhamnose were destroyed (216). Partial hydrolysis with *N*-acetic acid produced a degraded polysaccharide, which was still not dialyzable, and contained only traces of tyvelose. Oxidation of this product by periodate revealed that mannose was now oxidized, indicating that in the original polysaccharide tyvelose was linked to mannose (240).

By partial hydrolysis of the polysaccharide with 1 N H_2SO_4, many oligosaccharides were released, most of which contained rhamnose in a terminal reducing position. Three oligosaccharides were studied in detail (239*a*):

$$\begin{array}{l} \alpha\text{-Glc}(1 \rightarrow 4)\text{-Gal} \\ \qquad\qquad\quad \text{Gal} \rightarrow \text{Man} \rightarrow \text{Rha} \\ \alpha\text{-Glc}(1 \rightarrow 4)\text{-Gal} \rightarrow \text{Man} \rightarrow \text{Rha} \end{array}$$

During oxidation of the tetrasaccharide with periodate, galactose and mannose were destroyed. Since these hexoses are *not* periodate-sensitive in the intact molecule, it may be concluded that they are linked to another constituent in the native polysaccharide. As noted above, mannose carries a tyvelose residue. Galactose is considered to be linked to the rhamnose of another tetrasaccharide in the polysaccharide.

In analogy with the findings of Robbins and Uchida (176, 177) on the group E polysaccharides, these results led to the assumption that the group D_1 polysaccharides are composed of successive repeating units of identical oligosaccharides forming long chains, as shown in Table 8.

Salmonella of group B. S. paratyphi B (*1, 4, 5, 12*) and *S. typhimurium* (*1, 4, 5, 12*) polysac-

TABLE 8. *Repeating units of side chains isolated from acid hydrolysates of Salmonella O-specific polysaccharides and lipopolysaccharides*

Group	Repeating unit
D	α-glucose (1→4 or 1→6) tyvelose; → α-galactose → mannose → rhamnose →
B*	α-glucose (1→4 or 1→6) abequose (1→3); → α-acetylgalactose-(1 → 4)-β-mannose-(1 → 4)-rhamnose →
E_1	→ α-acetylgalactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
E_2	→ β-galactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
E_3	α-glucose (1→4); → β-galactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
E_4	α-glucose (1→6); → α-galactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
G	glucose-(1 → ?)- → [β-galactose-(1 → 3)-*N*-acetylgalactosamine-(1 → 3)-*N*-acetyl-galactosamine → fucose] →†
N	glucose (1→4); → [β-glucose-(1 → 3)-*N*-acetylgalactosamine → fucose] →†
U	α-galactose (1→3); → [β-galactose-(1 → 3)-*N*-acetylgalactosamine-(1 → 3)-*N*-acetylglucosamine-(1 → 4)-fucose] →†

* From *S. bredeney*.

† We do not know how the oligosaccharides of groups G, N, and U are linked in the side chains.

charides contain the same sugars as that of *S. typhi*, except that tyvelose is replaced by abequose. The amounts of monosaccharides recovered on hydrolysis did not account for 100% of the polysaccharide (170). For this reason, these polysaccharides have not been studied as extensively as that of *S. typhi*. However, the results of oxidation by periodate (239) and of partial hydrolysis (230, 98), performed as with *S. typhi*, showed that the polysaccharide of *S. typhimurium* was probably composed of repeating units for which the structure B in Table 8 was proposed (see also 4). However, in some serotypes of groups B and D (for instance, *S. typhi* T2 A.S.) glucose may be absent from repeating units, since the polysaccharides of these organisms contain only traces of glucose (95, 170).

Salmonella of groups E_1 (3, 10), E_2 (3, 15), E_3 (3, 15, 34), and E_4 (1, 3, 19). The structures of the first three subgroups have been investigated most thoroughly by Robbins and Uchida (176, 177), who studied products of methylation, oxidation, and partial hydrolysis. Recently, the polysaccharide of subgroup E_4 was also studied (225). Again, the sequence of sugars in the repeating units of these polysaccharides is similar to that of group B and D polysaccharides, but they are distinct by virtue of the linkages of the sugar units. For instance, throughout group E antigens, galactose is linked to carbon 6 of mannose, whereas in group B (*S. bredeney*) galactose is linked to carbon 4 of mannose (4). Moreover, the antigens of the four subgroups are differentiated by the presence or absence of glucose, by the anomeric position of galactose, and by the esterification of galactose with an acetyl group as shown in Table 8.

Salmonella of groups G (13, 22), N (30), and U (43). The polysaccharides of these three groups belong to chemotype VI (91). They are composed of the basal sugars with additional galactosamine and fucose. From partial hydrolysates, in addition to a number of smaller oligosaccharides, tetra- and pentasaccharides have been isolated, with the proposed structures shown in Table 8 (196). It is assumed that these oligosaccharides also represent repeating units, but it is not yet known how they are linked together to form the specific side chains.

The structure of *Salmonella* group U oligosaccharides offers a possible explanation for the known blood group B activity of this group. This structure includes a disaccharide unit, α-galactose-(1→3)-β-galactose-, which is also present in blood group B polysaccharide, and in which it plays the role of a determinant group for B activity (174). It seems possible that the oligosaccharide isolated from group G species represents an incomplete unit. Because of the known H(O) blood group specificity, it might be assumed that nonreducing fucose was originally present in the group G antigen in acid-labile glycosidic linkage which was split during hydrolysis (174).

Structure of O Factors in Specific Polysaccharides

Although the specificity of O antigens may not be due exclusively to the polysaccharide moiety of the somatic antigen complex (*see* 208, 210), there is no doubt that the degraded polysaccharide precipitates all those rabbit antibodies which, according to the Kauffmann-White scheme, agglutinate the species from which the polysaccharide was derived. For instance, the polysaccharide extracted from *S. paratyphi B (1, 4, 5, 12)* precipitated with antisera to *S. typhi (9, 12)*, *S. typhimurium (4, 12)*, and *S. senftenberg (1, 3, 19)*. It also precipitated with the homologous serum after the elimination of anti-*1*, anti-*4*, and anti-*12* antibodies, showing that the degraded polysaccharide contains at least part of the specificity characteristic of factor *5* (98). It is therefore concluded that the degraded polysaccharide exhibits the specificities (O factors) demonstrable by agglutination on the surface of the bacterial cell. These results were obtained when immunization of the rabbits was carried out so that antipolysaccharide precipitins are present in the sera. Short courses of immunization (2 weeks), which produce high titers of agglutinins, generally give only low titers of precipitating antipolysaccharide antibodies. On the other hand, after a second course 1 month later (23), precipitins are generally obtained in high titer.

Another important point is that the different O factors of a *Salmonella* serotype are *not* individual molecules, separable by chemical or serological fractionation, but these different O specificities are carried by one and the same polysaccharide molecule: the O antigen. This has been shown with the aid of specific serological methods in many systems with either the complex O antigen, the lipopolysaccharide, or the degraded polysaccharide (*see* 118). The specificity of an antigen is defined by the presence of multiple and discrete determinant groups (*see* 104, 128). Kabat (*see* 81) has shown with the aid of inhibition studies that a single determinant group of a polysaccharide may contain six or seven sugar units. It is often possible to inhibit the combination of antibodies with a determinant group A → A → A → A → A- or A → B → C → D → E → by prior incubation with sugar A, disaccharide A → A(A → B), or trisaccharide A → A → A(A → B → C), etc. The

closer the structure of the inhibitor is to the structure of the original determinant group, the more effective is the inhibition. Since no better inhibition was obtained with hexa- or heptasaccharide, Kabat concluded that in dextran the determinant group was of the order of a hexa- or a heptasaccharide, which fits previous data obtained with artificial antigens (*see* 81). Further, it has been known since the pioneering work of Landsteiner (*see* 104), Haurowitz (60, 61), and Heidelberger (*see* 68*a*) that a given determinant group produces a family of antibodies. Some of these antibodies can cross-react with other *similar not identical* determinant groups.

In the case of polysaccharides containing hexasaccharides as determinant groups, the different specific sites of the family of antibodies may be adapted to smaller oligosaccharides (penta, tetra, tri, di) possessing all the same terminal nonreducing sugars (47*b*, 191).

Since the different O factors of the Kauffmann-White scheme are detected by cross-agglutination or homologous agglutination with absorbed sera, they might consist of only parts of homologous determinant groups. Since they are present on polysaccharides, it was anticipated that they might be related to oligosaccharides of different lengths. At their maximum, these would be identical with one of the determinant groups of the O polysaccharide molecule.

To test this hypothesis, inhibition studies were carried out with different factor-antifactor systems. The degree of inhibition was measured by determining the amount of precipitate formed in the presence and absence of inhibitor, or by assaying the amount of complement fixed by the complex. However, complement is fixed only by the complex formed with Boivin antigen, lipopolysaccharides, or alkali polysaccharide and the corresponding antibodies. Degraded polysaccharide-antiserum complexes generally do not fix complement.

Role of 3,6-dideoxyhexoses: O factors 2, 4, 8, 9, 35, 50. The first studies relating the specificity of an O factor to the presence of a terminal sugar in a *Salmonella* polysaccharide appeared in 1956 (216). Serological analysis, by means of precipitation tests with specific polysaccharides derived from strains of group B (*4, 5, 12*) and D_1 (*9, 12*), before and after oxidation with periodate, revealed that most of the activity due to factor *12* was abolished at the same time that glucose and rhamnose were destroyed by periodate. On the other hand, the oxidized polysaccharides retained the specificity of factor *4* or *9*, respectively, and neither abequose nor tyvelose was destroyed. There appeared, then, to be a relation between factor *12* and the presence of glucose and rhamnose, between factor *4* and abequose, and between factor *9* and tyvelose. These inferences were confirmed by precipitation inhibition (217, 218), as glucose and rhamnose are the best inhibitors of the *12* anti-*12* system, abequose of the *4* anti-*4* system, and tyvelose of *9* anti-*9*. Similarly, colitose inhibited the best the precipitation of *S. adelaide* polysaccharide (factor *35*) with homologous antiserum.

Similar studies with other factors gave the results shown in Fig. 3, in which the specific sugars are drawn for simplicity at the end of lines, symbolizing determinant groups of the polysaccharide. Inhibition of precipitation was much more pronounced, when synthetic glycosides were used instead of the free sugars. In the case of 3,6-dideoxyhexoses, the α-anomers of abequose, tyvelose, and colitose exerted more inhibitory power than did the free sugars or the β-anomers (227, 228) (Table 9).

It has been demonstrated (see Tables 1 and 5) that O antigens containing the same sugars, and therefore belonging to the same chemotype, may nevertheless carry quite different specificities, i.e., they may belong to different serogroups. It is now clear (Fig. 3) that even the presence of an identical terminal sugar in two polysaccharides need not necessarily result in a common factor. For instance, both factors *4* and *8* contain terminal abequose, but they are serologically distinct. The same is true for factors *35* and *50*, both of which contain terminal colitose.

However, these findings were observed with highly specific rabbit antisera, such as those obtained after short immunization (slight cross-reactions may be observed after hyperimmunization of these animals). Horse antisera, on the other hand, exert a much broader specificity. For instance, a horse antiserum to *S. paratyphi B* (*1, 4, 5, 12*) precipitates the polysaccharide extracted from *S. newport* (*6, 8*). It was suspected that terminal abequose present on these polysaccharides was responsible, and it was indeed possible to inhibit this cross-reaction with abequose (218). Similar inhibition studies have shown that colitose, present in both *E. coli* O111 and *E. coli* O55 antigens, is responsible for the cross-reactions of these compounds with horse antisera (Staub, *unpublished data*). A similar relative lack of specificity was observed with goat antisera, as well as with a few antisera from hens, which showed cross-reactions similar to those seen with the horse sera even after a short immunization. It seems, therefore, that horses, goats, and some hens may form easily antibodies which are directed against one single terminal monosaccharide, whereas most antibodies pro-

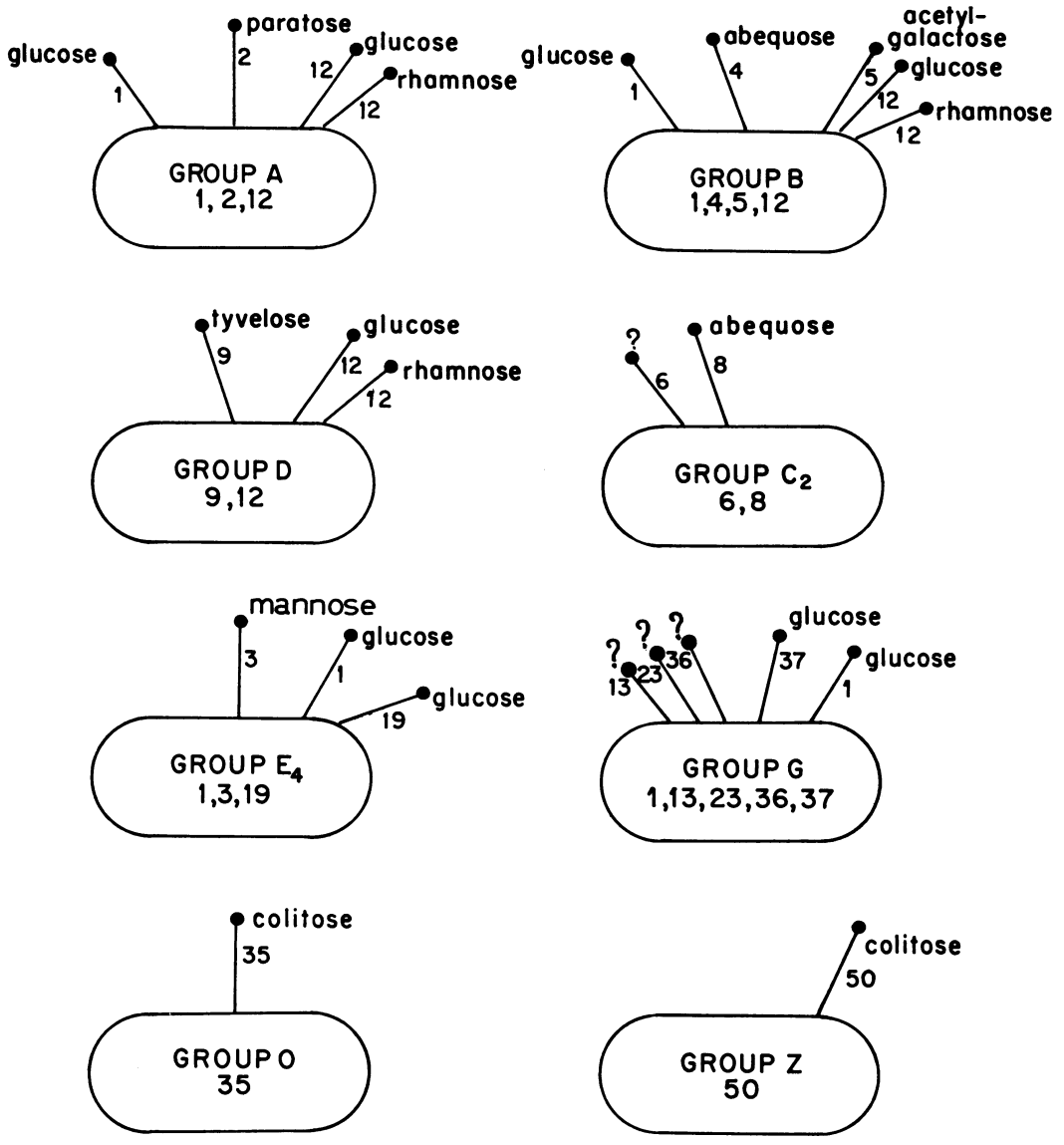


FIG. 3. *Schematic representation of sugars responsible for the specificity of some O factors of Salmonella as determined by serological inhibition studies. As noted below, these sugars need not be structurally terminal.*

TABLE 9. *Inhibition of precipitation by 3,6-dideoxyhexoses and their α- and β-glycosides (119, 227, 228)*

Inhibitor	Per cent inhibition of precipitation with anti-								
	9 (α-Tyvelose)			*4* (α-Abequose)			*O111* (α-Colitose)		
	0.4 μmoles	2 μmoles	10 μmoles	0.4 μmoles	2 μmoles	10 μmoles	0.4 μmoles	2 μmoles	10 μmoles
α-Glycoside	21	37	71	30	40	60	41	54	67
Sugar	10	22	45	—	18	27	5	18	31
β-Glycoside	7	15	34	2	5	25	0	0	8

duced by rabbits are directed against at least a disaccharide unit.

Artificial antigens. Further proof of the function of colitose as the determinant end group in *E. coli* O111 and cross-reacting O antigens was furnished by immunization with an artificial antigen containing colitose as the specific determinant group. For this purpose, an antigen was prepared in the same manner as the artificial antigens bearing mono- and disaccharidic determinant groups used by Goebel and Avery (53) in their studies of serological reactions.

Inhibition studies showed that α-aminophenyl colitoside was a better inhibitor than the β compound (Table 9). The α-glycoside was therefore coupled to bovine serum albumin and egg albumin. These artificial colitose antigens precipitated with *E. coli* O111 and *E. coli* O55 horse antisera (119). It was noticed, however, that in *E. coli* O111 antisera 50% of the antibodies to the homologous polysaccharide were precipitable by the artificial antigen, whereas only a few per cent of the homologous antibodies were precipitated from *E. coli* O55 antiserum. After elimination of the antibodies cross-reactive with *E. coli* O111 polysaccharide, no precipitation whatsoever of anti-*E coli* O55 horse serum by the artificial antigen could be observed, whereas the elimination of cross-reacting antibodies from *E. coli* O111 serum by *E. coli* O55 polysaccharide left most of the antibodies which were capable of reacting with the artificial antigen.

Immunization of rabbits with colitose linked to bovine serum albumin yielded high levels of anticolitose antibodies. These precipitated colitose egg albumin conjugate, and precipitation was inhibited up to 100% by colitose. However, bacilli containing terminal colitose in their polysaccharides were not agglutinated, in accord with the results of Goebel (50, 51) and McCarty (125), who also failed to obtain antibacterial antibodies in rabbits immunized with antigens containing only the single sugar forming the end group of the determinant specific oligosaccharide.

The ability of goats to produce less specific antisera was therefore used to obtain antibacterial agglutinins with the artificial antigen. Indeed, it was found that, although goats produced fewer anticolitose antibodies than did rabbits, these antibodies had a wider specificity; they agglutinated bacteria and precipitated the bacterial polysaccharide, resembling the *E. coli* O111 antisera obtained in goats after immunization with the microbes themselves (119).

Analogous studies with tyvelose and abequose, coupled to protein by diazotization of their *p*-aminophenyl-glycosides, gave similar results: good production of antidideoxyhexose antibodies in rabbits, with no antibacterial activities. Antibody production in goats was poor, but when it occurred, the antibodies agglutinated bacteria containing the corresponding sugar in their O antigen (228).

The data in Table 10 summarize the results obtained with goat antisera against colitose and tyvelose antigens. The "natural agglutinin" titers with bacteria lacking the dideoxyhexoses served as controls. After immunization, only the agglutinins related to the artificial antigen used in the immunization were elevated. These antibodies also exert opsonizing activity, as do the antibacterial antisera (Biozzi and Staub, *unpublished data*).

The absence of antibacterial antibodies in the sera of rabbits after immunization with artificial dideoxyhexose antigens was further demonstrated with anti-rabbit fluorescent antibodies. Bacteria having O antigens with the same 3,6-dideoxyhexose as the artificial antigen, when preincubated with antidideoxyhexose rabbit serum, did not fix the fluorescein-conjugated anti-rabbit antibodies. It is concluded that the specific sites of rabbit anticolitose antibodies, for example, are closely adapted to at least the phenyl colitoside and cannot fit the terminal disaccharide present on the microbial polysaccharide. On the other hand, some goat antibodies either fit the phenyl colitoside less closely or are adapted to the terminal colitose only, and therefore can form a complex with the microbial polysaccharide.

Factor 5. The nature of factor *5* was established by Kotelko, Staub, and Tinelli (98) in studies of mutants of *S. typhimurium* with and without this factor. With partial hydrolysates of the respective polysaccharides, no differences could be detected on paper chromatograms. However, when oligosaccharides were studied in inhibition tests with the *5* anti-*5* system, one oligosaccharide derived from *S. typhimurium* polysaccharide containing factor *5* was strongly inhibitory. Chemical studies showed that, in contrast to the analogous oligosaccharide derived from the polysaccharide without factor *5*, this active oligosaccharide contained *O*-acetyl groups. Both active and inactive oligosaccharides were hexasaccharides containing one terminal nonreducing galactose and one terminal reducing rhamnose. The sequence of the sugars in this hexasaccharide has not been established, but our present knowledge concerning the repeating unit of group B chains (see Table 8) allows the assumption that the hexasaccharide represents a chain of two repeating galactose $\rightarrow$ mannose $\rightarrow$ rhamnose units (Table 11). Although inhibition studies with acetylgalactoses could not be performed, because these sugar derivatives were not available, it was shown that *N*-acetyl-D-galactosamine was a rather good inhibitor of the *5* anti-*5* system, being much more effective than

TABLE 10. *Agglutinin titers after immunization of goats with artificial antigens (119, 227, 228)*

Bacteria agglutinated	Anticolitose serum*		Antityvelose serum*	
	Before immunization	After immunization	Before immunization	After immunization
Strains containing colitose				
Echerichia coli O111 B4	<100	800	—	—
E. coli O55 B5	<100	400	—	—
Salmonella-adelaide	20	400	—	—
S. greenside	40	400	—	—
Arizona O9	40	400	—	—
Strains containing tyvelose				
S. typhi	—	—	<50	200
S. haarlem	—	—	100	400
Pasteurella pseudotuberculosis	—	—	<10	50±
Strains containing neither colitose nor tyvelose				
E. coli O25	320	400	50±	50
S. abortus equi	20	<100	<50	50±
S. senftenberg	—	—	200	200
S. cholerae suis	<100	<100	—	—
S. paratyphi A	—	—	<50	<50

* Reciprocal agglutinin titers.

TABLE 11. *Oligosaccharides responsible for the specificity of various O factors as demonstrated by inhibition techniques*

Factor	Group	Oligosaccharide	Reference
	B		Stocker et al. (230)
1	E_4 G	α-Glucose-(1 → 6)-galactose	Staub and Girard (225)
1_{12}	B	α-Glucose-(1 → 6)-galactose → mannose → (rhamnose) *→	
1_{19}	E_4	α-Glucose-(1 → 6)-galactose-(1 → 6)-mannose-(1 → 4)- rhamnose →	Staub and Girard (225)
37	G	α-Glucose-(1 → 6)-galactose → X†	
5	B	Acetylgalactose → (→mannose → rhamnose → galactose → mannose →)‡ → rhamnose →	Kotelko, Staub, and Tinelli (98)
3	$E_{1,2,3,4}$	Mannose → rhamnose → galactose →	Uchida, Robbins, and Luria (24)
10	E_1	α-Acetylgalactose → mannose → rhamnose →	Staub and Girard (225)
15	E_2	β-Galactose → mannose → (rhamnose) *→	Robbins and Uchida (177)
34	E_3	α-Glucose-(1 → 4)-β-galactose-(1 → 6)-mannose →	Uchida, Robbins, and Luria (241)
12_2	D	α-Glucose-(1 → 4)-α-galactose → mannose → (rhamnose) *→	Tinelli and Staub (240)

* The presence of this sugar on the factor is not certain.
† X = undetermined sugar.
‡ Order of the sugars not determined.

galactose. As galactosamine is not a constituent of the polysaccharide, these results seem to indicate that the *O*-acetyl group occupies the position 2 of galactose in the specific polysaccharide, and that *N*-acetylgalactosamine, because of its similar structure, is able to substitute for 2-*O*-acetylgalactose as inhibitor of the *5*-specific precipitation.

Factors 1, 1_{12}, 19, 37. Factor *1* specificity is common to a number of serotypes belonging to different serogroups, in which it is very often associated with that of another factor specific for a single group. This is apparent from the Kauffmann-White scheme for group E_4 (**1**, *3*, **19**), group *G* (**1**, *13*, *23*, *36*, **37**), and group T (**1**, *42_1*, **42_2**), but it is also true for group B, in which factor *1* can be differentiated into the specificities *1* and *1_{12}*.

Table 11 summarizes the conclusions drawn from results of chemical and serological analyses carried out by Staub and co-workers (209, 219, 225, 230) with split products of specific polysaccharides. These results agree very well with those obtained by Iseki and co-workers (75), who concluded from inhibition studies with simple sugars, glycosides, and disaccharides that factor *1* was terminated by an α-glucosyl residue linked to carbon 6 of the following sugar.

For inhibition of the E_4 system, tests were made with a number of oligosaccharides, which had been obtained from partial hydrolysates of *S. senftenberg* polysaccharide. Figure 4 II shows that the tetrasaccharide is by far the best inhibitor of the *19* anti-*19* system, suggesting that at least four sugars are involved in the specificity of factor *19* (225).

In contrast to these results, it is clear from Fig. 4 I that anti-*1* antibodies of the same anti-E_4 serum are maximally inhibited by the disaccharide α-glucose-(1→6)-galactose. The tri- and tetrasaccharides are not more efficient. On this basis, it has been proposed (225) that factor *1* of group E_4 is limited to the terminal disaccharide α-glucose-(1→6)-galactose of the tetrasaccharide carrying the specificity of factor *19*. Similar studies carried out with factors *1* and *1_{12}* of group B and *1* and *37* of group G lead to analogous conclusions. From the results obtained with factors *1*, *1_{12}*, *19*, and *37*, it is concluded that the common factor *1*, present in each group, is not a distinct structure in the respective polysaccharide different from the structure of factors *1_{12}* in group B, *19* in group E_4, or *37* in group G, but that factor *1* represents a common terminal, nonreducing disaccharide which is part of each of these factors. This explains why factors *1* and *19*, or *1* and *37*, are always present simultaneously in the bacteria. We assume that a similar principle exists in other groups of the Kauffmann-White scheme, in which factor *1* is present, for instance, in group R (*40*).

Factors 3, 10, 15, and 34. Studies carried out on factors *3*, *10*, *15*, and *34* (176, 177, 241) provided the results summarized in Table 11. It can be seen that the specificity of factor *10*, like that of factor *5*, is determined by an *O*-acetylgalactose. Robbins and Uchida did not isolate the corresponding acetylated oligosaccharide, but the inability of the oligosaccharides isolated from

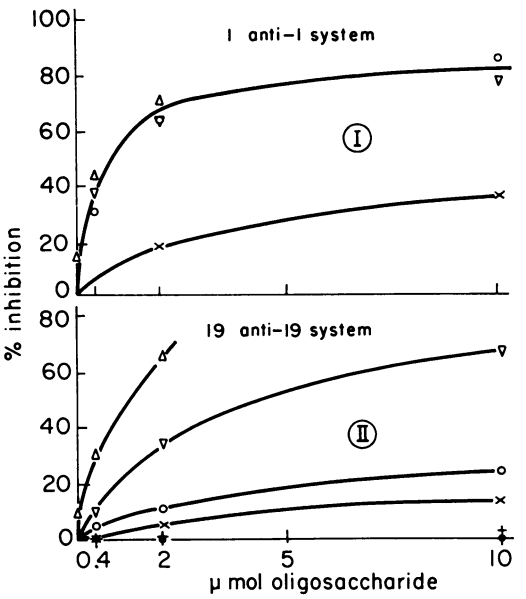


FIG. 4. *Inhibition of 1 anti-1 and 19 anti-19 systems by oligosaccharides extracted from Salmonella E_4 (1, 3, 19) polysaccharide [after Staub and Girard (225)]. (I) Antibodies anti-1 present in a rabbit anti-E_4 (1, 3, 19) serum precipitated with Salmonella B (1, 4, 12) polysaccharide. (II) Antibodies anti-19 present in the same rabbit anti E_4 (1, 3, 19) serum obtained by elimination of anti-1 by the system (I) and anti-3 by precipitation with an E_1 (3, 10) polysaccharide. Precipitation by the homologous E_4 (1, 3, 19) polysaccharide. Symbols:* ●, *glucose;* ×, α-methyl glucoside; +, β-methyl glucoside; ○, *disaccharide α-glucose-(1→6)-galactose;* ▽, *trisaccharide α-glucose-(1→6)-galactose;-(1→6)-mannose;* △, *tetrasaccharide α-glucose-(→6)-galactose-(1→6)-mannose→rhamnose.*

partial hydrolysates to inhibit anti-*10* antibodies led the authors to suspect the presence of a labile constituent as part of factor *10*. They found that the specificities *10* and *5* were destroyed by alkali at the same rate, and demonstrated the presence of *O*-acetyl groups in the E_1 specific polysaccharide. In addition, brief acetylation of the serologically inactive trisaccharide α-galactose →mannose→rhamnose converted it into a potent inhibitor of the *10* anti-*10* system. The

mild conditions of acetylation indicated that in factor *10* galactose carries the acetyl group in position 6, unlike factor *5* in which galactose is probably acetylated in position 2.

It is very probable that the specificities of factors *3*, *10*, *15*, and *34* are also determined by at least four sugars, although no inhibition studies have been performed with tetrasaccharides. With respect to factor *3*, the acid lability of the linkage between rhamnose and galactose in the polysaccharide prevents the formation during partial hydrolysis of reasonable amounts of oligosaccharides containing this linkage (for instance, mannose → rhamnose → galactose). It has been shown, however, that factors *3* of group E_1 and E_4, whose polysaccharides contain α-linked galactose, are more like each other than like factor *3* of group E_2, in which galactose is linked β-glycosidically (109, 225). It is therefore concluded that galactose plays a role in the specificity of the different factors *3*. This shows again that the Kauffmann-White scheme is a simplified scheme and, as predicted by Kauffmann (88), most of the O factors can be subdivided.

Factor 12. Inhibition studies with simple sugars showed that anti-*12* rabbit antibodies were best inhibited by glucose; with horse antibodies, inhibition was obtained always with rhamnose and, in some sera, also with glucose. These results justified the two chains drawn in Fig. 3. It was also found that rhamnose was an inhibitor of the unexpected cross-reaction between anti-*S. senftenberg* horse serum (*1*, *3*, *19*) and the specific polysaccharide of *S. typhimurium* (*4*, *12*). Since this precipitation does not involve factor *12*, as defined by the use of rabbit sera, the role of rhamnose in the specificity of factor *12* is not quite clear. On the other hand, it should be noted that the only sugar missing in *Citrobacter* (*4*, *5*), which cross-reacts with *S. paratyphi* B (*1*, *4*, *5*, *12*), is rhamnose. The same is true for the cross-reacting pair of *P. pseudotuberculosis* II (*4*, *27*) and *S. schleissheim* (*4*, *12*, *27*) (see Table 7) (96*a*, 258) in which again both factor *12* and rhamnose are missing in the *Pasteurella* strain.

Factor *12* has been divided into three subfactors: 12_1, 12_2, and 12_3 (88). Of these, only factor 12_2 has been studied extensively by Tinelli and Staub (239*a*): it is one of the determinants with glucose as end group and consists in part or completely of the tetrasaccharide, α-glucose-(1→4)-galactose→mannose→rhamnose.

According to the results discussed above, two factors possessing an identical terminal disaccharide should give cross-reactions in rabbit sera. Thus, factors *34* and 12_2, which carry the same terminal α-glucose-(1→4)-galactose disaccharide, should cross-react. This does in fact occur, since Kauffmann observed that there was some cross-agglutination between bacteria possessing factors *34* and 12_2 (88). However, the fact that galactose is linked α-glycosidically in factor 12_2 and β-glycosidically in factor *34* results in a relatively weak cross-reaction compared with the strong cross-reaction between factors 1_{12} and *19*. Therefore, no common factor between groups E_3 (factor *34*) and B (factor 12_2) appears in the Kauffmann-White scheme.

It appears, therefore, that some O factors of the Kauffmann-White scheme are related to oligosaccharides containing at least four sugar units which constitute the different determinant groups present in the somatic polysaccharides. Other factors are related to smaller oligosaccharides present in the same determinant groups; they are the expression of cross-reactions between two partly different determinant groups. However, O factors can never be related to oligosaccharides smaller than a disaccharide. Indeed, rabbit antisera obtained after short immunization are used to construct the classification in the Kauffmann-White scheme, and we have seen that such sera do not contain antibodies adapted to only one sugar (see above).

Location of the factors on the chain. The question arises as to where these factors are situated in or on the polysaccharide. According to our scheme (Fig. 1), in connection with the available chemical data, two possibilities might be offered: either (i) each factor is situated at the end of a distinct side chain or (ii) each individual chain carries all of the factors (Fig. 5). It was originally believed that the factors were present only at the ends of side chains and that the different factors were carried by different chains ending with different sugars (Fig. 5A). Experimental data showed that only a few antibody molecules are fixed per molecule of polysaccharide or lipopolysaccharide, and chemical investigations with Freeman type (degraded) polysaccharide extracted from *S. typhi* indicated that each constituent sugar might be present partly as a terminal nonreducing end group (237). However, Uchida et al. (241) demonstrated that, although mannose is the best monosaccharide inhibitor of the *3* anti-*3* system (225), the two oligosaccharides α-galactose→mannose→rhamnose and β-galactose→mannose→rhamnose are both able to inhibit this system to the same extent as, or even better than, the disaccharide mannose →rhamnose. Uchida and Robbins (*unpublished data*) also report that in the *34* anti-*34* system, in which glucose is the best monosaccharide inhibitor, the heptasaccharide

Gal → Man → Rha → Gal → Man → Rha
↑
Glc

is a better inhibitor than the tetrasaccharide

galactose → mannose → rhamnose
↑
glucose

Salmonella antibodies can thus be fixed on the chains of specific *Salmonella* polysaccharides (Fig. 5B) like antipneumococcal antibodies are on the linear chains of the capsular polysaccharides of types VI (173) and III (126). In such determinant groups present on a long chain, it is thus recognized that one distinct nonterminal sugar plays the same role in immunological specificity as a terminal nonreducing sugar; i.e., it possesses the highest affinity for the corresponding antibody as determined by inhibition tests. We propose to call these sugars, whether or not they are present at the end of the chain, *immunodominant sugars*.

It must be stressed, however, that because of steric hindrance very few antibodies can bind to one molecule of *Salmonella* polysaccharide. Only the factors present near the end of the side chains are available to antibodies. The space between the oligosaccharide side chains is probably too narrow (see discussion) to allow the antibodies to reach the factors situated near the central core.

In conclusion, it is certain that *Salmonella* O factors, as defined by the Kauffmann-White scheme, are related to short oligosaccharides containing two to four or more sugar units, which are carried on the specific long chains or at their extremities according to the general formulas

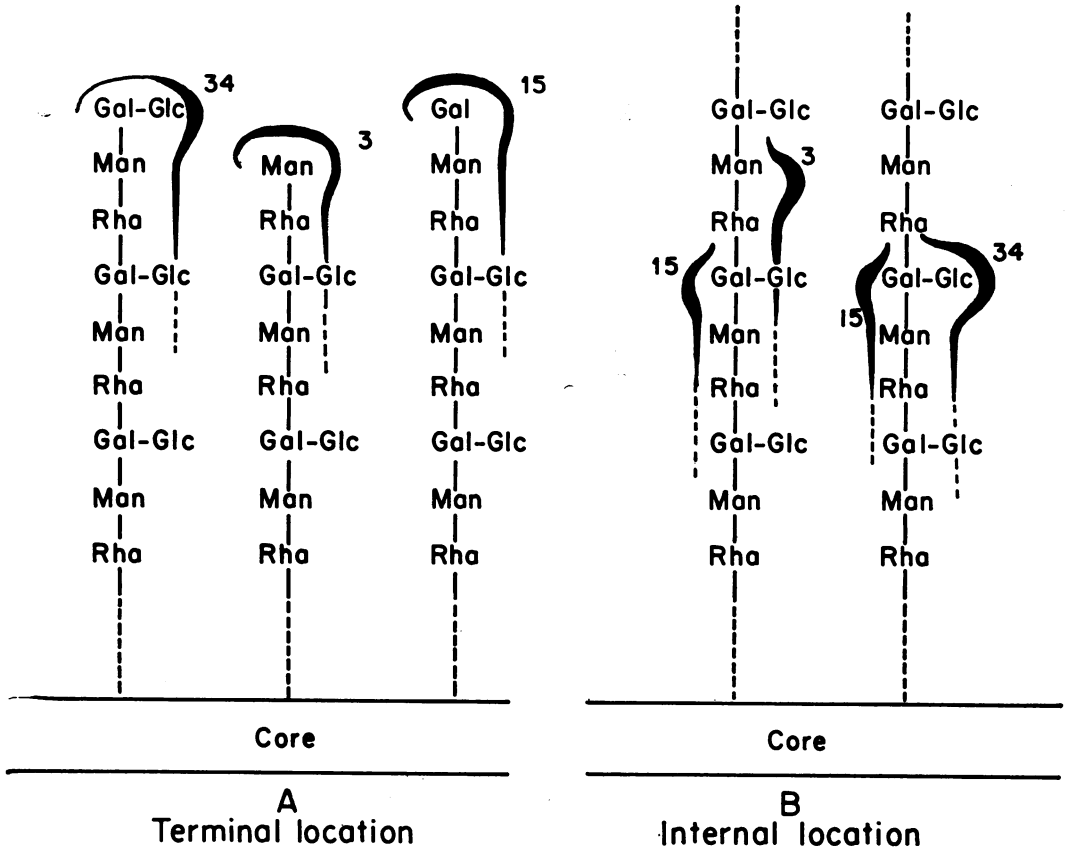


FIG. 5. *Possible location of factors on the side chains of Salmonella group E₁ (3, 15, 34) polysaccharides. The drawing around the chains (like those on Fig. 9 and 18) symbolizes the intensity of the affinity between the antibody combining sites and the sugars of the determinant groups present on the polysaccharide. The thicker the line the stronger the affinity. The strongest affinity occurs between the antibody and the "immunodominant" sugar, e.g., the sugar which best inhibits the corresponding antibody (see text).*

$$\rightarrow A \rightarrow B \rightarrow C \rightarrow D \rightarrow \quad (I)$$

or

$$\begin{array}{l} \quad A \\ \quad \downarrow \\ \rightarrow B \rightarrow C \rightarrow D \rightarrow \end{array} \quad (II)$$

in which the letters represent different sugars, and *A* acts as the immunodominant sugar. Changes of specificity (appearance of new factors) are created by the addition of a new sugar on the chain, for instance,

$$\begin{array}{l} \quad X \\ \quad \downarrow \\ \rightarrow A \rightarrow B \rightarrow C \rightarrow D \rightarrow \end{array}$$

and any structural alteration within the chain, such as replacement of one sugar by another, or change in the nature of a glycosidic linkage, including the linkage of the sugar preceding A in formula I or B in formula II.

many other *Salmonella* factors have been related to the presence of a prophage (Table 12; *see* 6, 113). It has also been shown that the phage need not be in the form of a prophage to produce the new factor. The latter can be detected as early as 8 min after phage infection (109, 243, *see also* 6), an interval during which the genetic material is certainly not yet incorporated into the genome of the bacteria. The appearance of such factors is often accompanied with form variation, so that, to obtain crops of bacteria rich in such factors, the organisms must be grown in the presence of the phage (de Margerie *unpublished data in* 230).

Serological and bacteriological studies summarized in Table 12 have shown that conversion by phage can result in three different changes: one new factor appears (for instance factors *20, 34*), two new factors appear (factors *1*, 1_{12} ; *1, 37*; *1*, 42_2 ; *27, 27*A), or one factor is replaced by another factor (factor *10* → *15*).

TABLE 12. *Change in serological specificity due to conversion of Salmonella by phages*

Salmonella group	Phage	Change involved	Reference
E	ξ15	*3, 10* → *3*, **15**	Iseki and Sakai (71)
	ξ34	*3, 15* → (*3*), (*15*), **34**	Uetake et al. (242, 243) Harada (62, 63)
D_2	ξ15	*3, 9, 46,* → *3*, **15**, *9, 46*	Le Minor (109)
	ξ34	*3, 15, 9, 46* → (*3*), (*15*), **34**, *9, 46*, $\mathbf{12_2}$	Le Minor (109)
A	Iota	*2, 12* → **1**, *2, 12*	Iseki and Kashiwagi (72)
B	Iota	*4, 12* → **1**, *4, 12*	Zinder (268), Stocker (229)
	P22		Iseki and Kashiwagi (72)
D	Iota	*9, 12* → **1**, *9, 12*	Iseki and Kashiwagi (72)
A	φ27	*2, 12* → *2, 12*, **27A**, **27**	Le Minor (107), Le Minor, Le Minor, and Nicolle (113)
B	φ27	*4, 12* → *4, 12*, **27B**, **27**	Le Minor (107)
D	φ27	*9, 12* → *9, 12*, **27D**, **27**	Le Minor (107)
C_2	φ20	*6, 8* → *6, 8*, **20**	Baron, Formal, and Washington (7)
C_1	$\varphi(6_1)$	*6, 7* → *6*, 6_1, *7*	Escobar and Edwards (34)
C_1	φ14(6, 7)	6, 7 → *6*, (*7*), (**14**)	Le Minor (111)
K	φ6, 14, (18)	*18* → **6**, **14**, *18*	Le Minor (110)
G	φ37	*13, 23, 36,* → **1**, *13, 23, 36*, **37**	Le Minor, Ackermann, and Nicolle (112)
R	φ40	*40* → **1**, *40*	Le Minor (108) and *personal communication*
T	φ42	42_1 → **1**, 42_1 , $\mathbf{42_2}$	" "

Biochemical and Genetic Basis of Changes in Specificity After Phage Conversion

Iseki and Sakai (71) showed for the first time that the existence of some factors was due to the presence of a phage in the bacteria. The studies of Iseki were concerned with factor *15*. Since then, Generally, a given phage is specific for a given group. For instance, phage 40 converts members of *Salmonella* serogroup R (*40*), but not serotypes of group T (*42*), although factor *1*, which appears after conversion by phage 40, is also present in serotypes of group T after phage conversion. But in this latter group conversion is

due to phage 42, which, in turn, cannot convert bacteria of group R (108). On the other hand, serotypes belonging to serogroups A, B, and D_1 can be converted by identical phages, possibly because their cell wall polysaccharides are very similar and therefore may possess the same specific receptor sites. Although phages 27 and P22 both convert bacteria of groups A, B, and D_1, the results are quite different (Fig. 6). Phage P22 produces a new factor, factor *1* (or factors *1*, 1_{12}), which is identical or nearly so in members of groups A, B, or D_1 (108); conversion by phage 27 provokes the formation of a common factor

α-glucose-(1 → 6)-galactose

α-glucose-(1 → 6)-
galactose → mannose → rhamnose

Later, a third oligosaccharide, namely, the trisaccharide galactose→mannose→rhamnose, was obtained from the polysaccharide of the converted strain after prolonged hydrolysis, as well as from the parent strain polysaccharide after short hydrolysis (219). The liberation of this oligosaccharide under different conditions of hydrolysis is due to differences in acid stability of the glucose-(1→4)-galactose and glucose-(1→

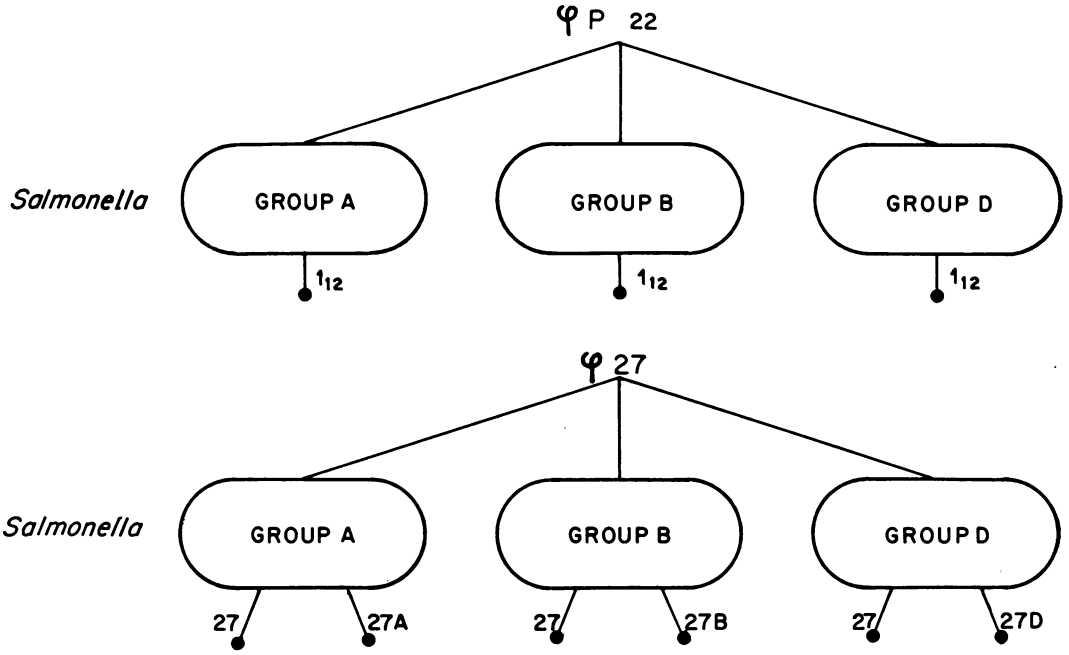


FIG. 6. *Scheme of conversion of groups A, B, and D Salmonella by phages P 22 and 27 [after Staub and Raynaud (221)].*

27, which, however, is always produced in combination with a second factor, *specific* for each group: *27*A, *27*B, and *27*D (107).

Changes of specificities also appear after conversion of *Shigella* by phage (*see* 113). Immunochemical investigation of these has been begun by Itikawa (76).

Appearance of factor 1. Stocker et al. (230) made the first chemical analysis of such lysogenic conversion with a *S. typhimurium* strain (*4, 12* not containing factors *1* and *5*) and phage P22. Two oligosaccharides which were absent from the polysaccharide of the wild-type strain could be isolated from partial hydrolysates of the polysaccharide of the converted strain:

6)-galactose linkages present, respectively, in the wild and the converted type tetrasaccharide (230, 239*a*) α-glucose→galactose→mannose→ rhamnose. The 1→6 linkage in the converted strain is much more stable to acid and delays the formation of the trisaccharide galactose→mannose → rhamnose. In this case, phage P22 supplies the genetic information necessary for the appearance (or derepression) of an enzyme which links the short side chain, glucose, to the carbon 6 of galactose present on the long chain of the wild-type polysaccharide, as suggested by Staub (209).

The specific structure of factor 12_2 is given by terminal glucose bound α-(1→4)- to galactose in the main chain (see Table 8). It was suggested,

therefore, that in the presence of the phage either there is a competition for the formation of factors *1* and *12*$_2$ (α-glucose-(1→6)-galactose → and α-glucose-(1→4)-galactose→), or there is a repression of the enzyme which transfers glucose to the 4 position of galactose. Staub (*unpublished data*) showed that precipitation of anti-*12* antibodies was greatly diminished when the polysaccharide carried factor *1* (Fig. 7).

Although structural comparison of wild-type and converted-type polysaccharides in group G has not been achieved, the results obtained by inhibition studies with the disaccharide α-glucose-(1→6)-galactose (225) suggest that in this group the phage provokes the manifestation of the enzyme which again links α-glucose to carbon

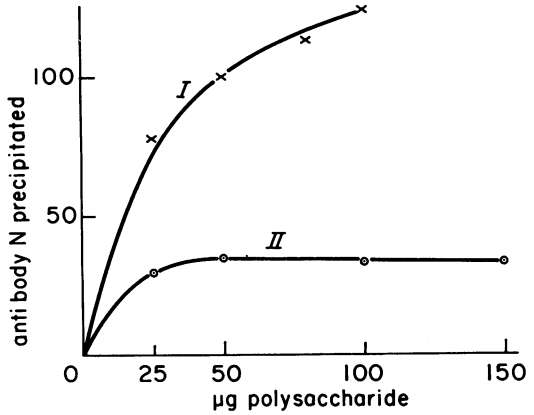
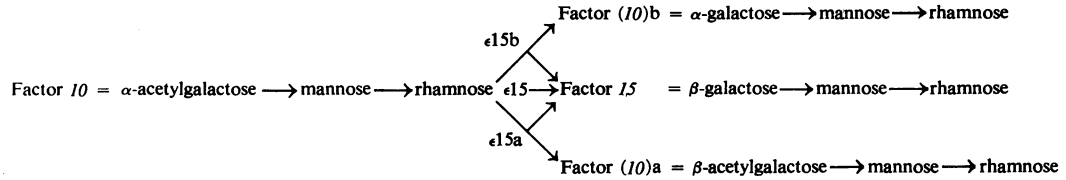


FIG. 7. *Illustration of competition between factors 1 and 12*$_2$. *Precipitation of 0.5 ml of a horse anti-Salmonella typhi (9, 12) serum by polysaccharides extracted from S. typhimurium (4, 12) (curve I) and S. bredeney (1, 4, 12) (curve II).*

6 of the galactose present on a pre-existing unit α-galactose → X → Y →, leading to the simultaneous formation of factor *1* (α-glucose-(1→6)-galactose) and factor *37* (α-glucose-(1→6)-galactose → X → Y).

Conversions in group E. From the work of Robbins and Uchida (176, 177, 241), it is known that phage ϵ34, which is responsible for the appearance of factor *34* in group E_2, plays a role analogous to that of the phages discussed above. It provokes the formation (or derepression) of an enzyme which links α-glucose to carbon 4 of the β-galactose present in the chain; this results in conversion of factor *15* into factor *34*. The same authors studied the chemical changes occurring when factor *10* is converted into factor *15* by phage ϵ15. Here the phage produces the change of an *O*-acetyl-α-galactose group, responsible for the specificity of factor *10*, into a non-acetylated β-galactosyl group which determines the specificity of factor *15* (see Fig. 8a).

It is now possible to understand why a serotype of group E_1 (*3, 10*), lysogenized by phage ϵ34 cannot produce factor *34*: it lacks the β-galactose which is necessary for the attachment of glucose. As soon as the α-galactose is replaced by the β-galactosyl residue, i.e., after superinfection by phage ϵ15, factor *34* can appear as shown in Fig. 8a. The genetic information carried by phage ϵ15 obviously is more complex than in the case of the phages responsible for the presence of factors *1* and *34*; the activity of one enzyme appears, while the activities of at least two enzymes disappear. In studying the mechanisms of this change, Robbins et al. (179, 180) showed that after conversion the acetylating enzyme was no longer detectable. Furthermore, mutants of phage ϵ15 were isolated whose action was less complex. Mutant ϵ15b suppressed (or repressed) only the acetyl transferase, and ϵ15a suppressed (or repressed) only the α-galactose transferase with simultaneous formation (or derepression) of the β-galactose transferase. Bacteria, converted by these phage mutants, carried a factor (*10*) (the factor in brackets cross-reacts, but is not identical, with the factor without brackets) which cross-reacted with the true factor *10*, but both factors (*10*), [(*10*)a and (*10*)b], were different. The following chemical relationships were established:



Conversion by phage ϵ15b greatly reduces the number or length of specific side chains, as does conversion by a third mutant, ϵy, which does not induce the formation of factor *15* (179).

Appearance of factors 27A, 27B, and 27D in groups A, B, and D after conversion by phage 27. Since no immunochemical investigations have been carried out on the factor *27* common to the three groups A, B, and D_1 (see Fig. 6), only the factors specific for each group, *27*A, *27*B, and *27*D, will be discussed.

The appearance of three different factors *27*A,

*27*B, and *27*D in *Salmonella* serotypes of groups A, B, and D_1 converted by the same phage seems at first glance difficult to relate to a mechanism analogous to that just discussed. The initial experimental data (220) showed that each of the new factors contains the terminal 3,6-dideoxyhexose 4_2, and 9_2, changed by conversion into *27*A, *27*B, and *27*D, respectively.

Chemical analyses of oligosaccharides of the wild and converted strains of group B showed (3, 4) that the only detectable difference after conversion was the presence of galactose-(1→6)-man-

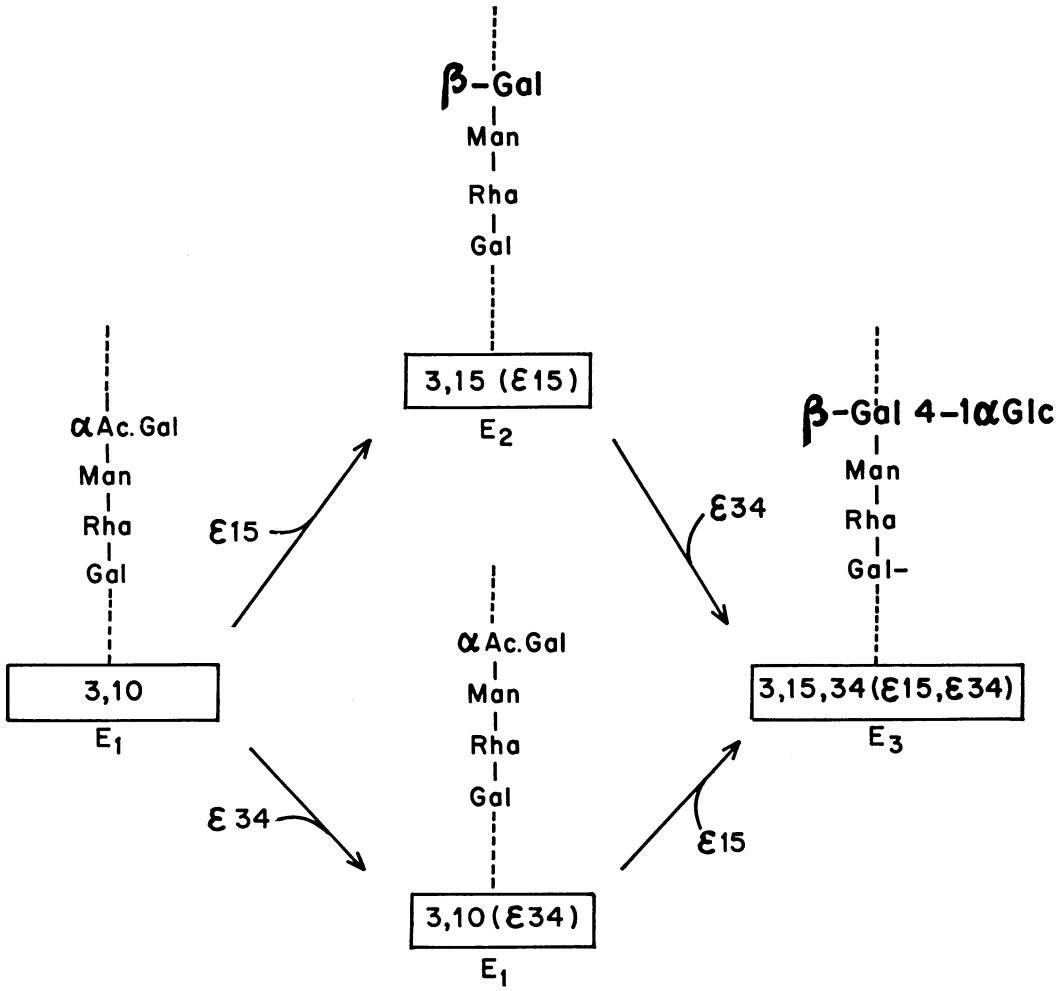


FIG. 8a. *Lysogenic conversions in Salmonella group E [based on data of Uchida, Robbins, and Luria (241)].*

specific for the serogroup to which it belongs. Further immunological studies (3; Staub and Bagdian, *unpublished data*) demonstrated that factors *2*, *4*, and *9* could each be divided into two subfactors, of which only one is present after conversion (Table 13).

From the results obtained with factors *1*, one might postulate that factors 2_1, 4_1, and 9_1 (common to the wild and converted strains) were terminal di- (or tri-) saccharide units of longer oligosaccharides which carried specificities 2_2, nose instead of the galactose-(1→4)-mannose present in the wild strain. According to these data and the formula proposed in Table 8 for the chain of group B, we can visualize the change due to the phage as indicated in Fig. 9. The difference of specificity between *27*B and 4_2 could then originate either from the change of the galactose → mannose (I) linkage following abequose or from the change of the same linkage (II) preceding abequose. The chemical data rule out the first

hypothesis of a change of linkage between mannose and rhamnose (220) or of the anomeric position of rhamnose (221).

More experimental data are needed, but it seems possible that the complexity of the changes involved in conversion with phage 27 could be explained by a change in enzyme equipment which is very similar to the changes occurring in other conversions: appearance of a new enzyme linking galactose to carbon 6 of mannose and repres-

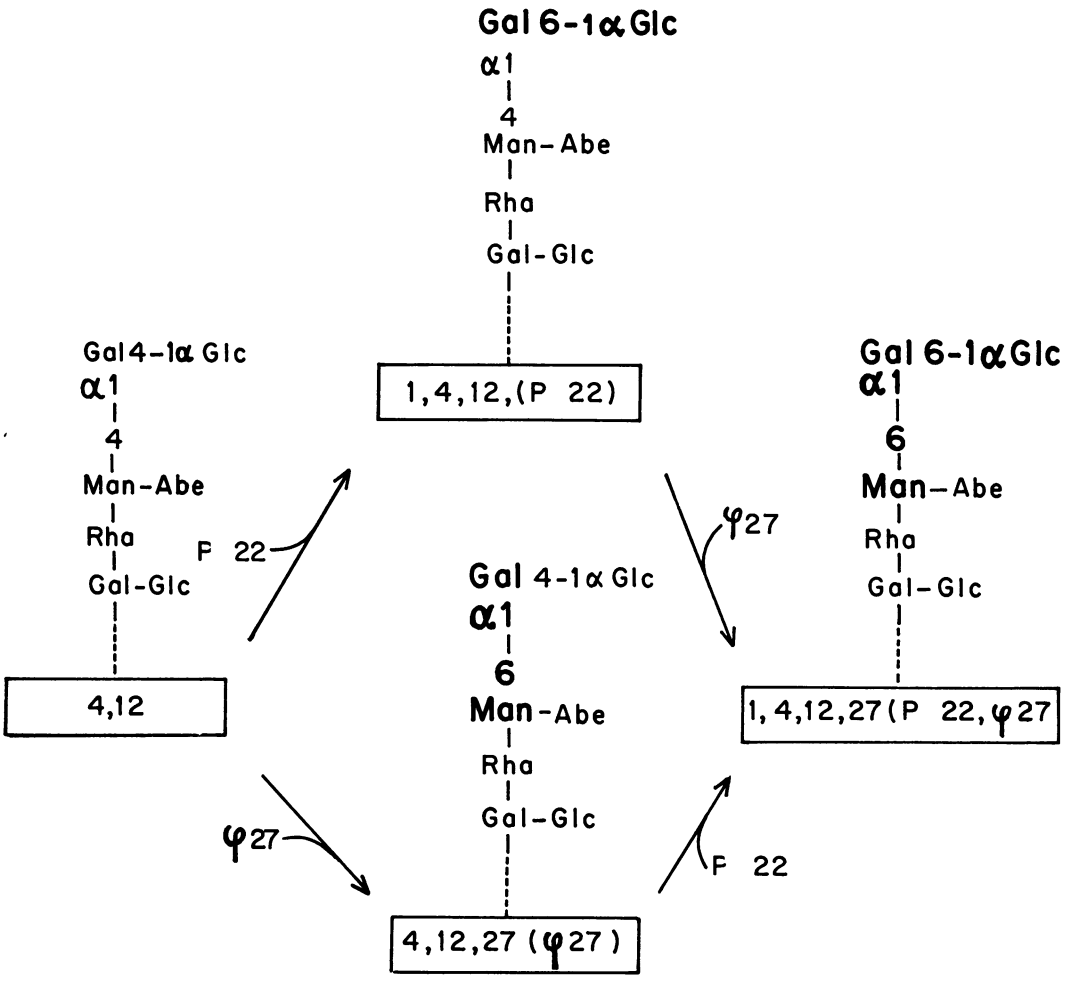


FIG. 8b. *Lysogenic conversions in Salmonella group B [based on data of Stocker et al. (230), Staub and Fores (220), and Bagdian (3)].*

TABLE 13. *Immunological modifications related to conversion of Salmonella by phage 27**

Group	O factors of the Kauffmann-White scheme	Wild strain			Converted strain	
		Newly established subfactors	Immunodominant sugars of the two subfactors		Subfactors	Immunodominant sugars of the two subfactors
A	2	2_1 2_2	Paratose	$\varphi 27$ →	2_1 27_A	Paratose
B	4	4_1 4_2	Abequose		4_1 27_B	Abequose
D	9	9_1 9_2	Tyvelose		9_1 27_D	Tyvelose

* For structural details see Fig. 9.

sion of the wild-type enzyme linking galactose to carbon 4 of mannose (see Fig. 8b).

As pointed out, the presence of phage $\epsilon 15$ in group E serotypes is necessary for the expression of antigen *34* by phage $\epsilon 34$ (Fig. 8a). In contrast (Fig. 8b), as expected from the biochemical results, the sequence of infections with phages 27 and P22 is not important, since the chemical changes produced by the two phages are independent.

STUDIES ON R ANTIGENS

It was shown in the first section that the long side chains of *Salmonella* O-antigenic polysaccharides are composed of repeating units of more or less complex oligosaccharides which carry the specificity of the O factors. These side chains were assumed to be linked to a basal core polysaccharide, common to all *Salmonella* O antigens.

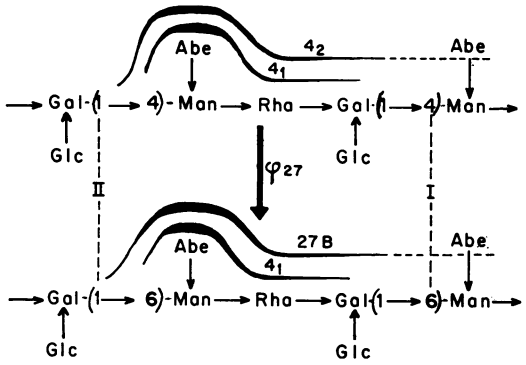


FIG. 9. *Chemical modifications linked to conversions of group B Salmonella by phage 27.*

This hypothesis was based on studies of chemical changes in the specific enterobacterial cell wall polysaccharide which occurred during S-R mutation. Instead of the O-specific polysaccharide, R mutants synthesize incomplete polysaccharides of lower chemotypes with R specificity. Therefore (27, 93, 117, 150), R specificity is associated with an internal underlying structure of the parent O-specific polysaccharide, and the more simple R polysaccharides of R forms represent the core of the more complex O polysaccharides of the S forms. This section deals with *Salmonella* R forms which are characterized by a defect in the biosynthesis of the cell wall polysaccharide of the (wild-type) S form.

According to present knowledge, the biosynthesis of O-specific polysaccharides occurs in two principal steps. The monosaccharide constituents of the antigen are manufactured in the form of their activated derivatives, generally as nucleoside diphospho sugars. With the aid of specific transferases, the sugar residues are transferred to the growing polysaccharide acceptor in a specific, genetically determined sequence.

Figure 10 shows the pathway of a number of sugar nucleotides which are intermediates in the biosynthesis of *Salmonella* and related O antigens. The central position of glucose and mannose as intermediates in the biosynthesis of many monosaccharides is apparent. Epimerization and reduction are the essential reactions leading to the deoxy sugars. In most reactions, uridine diphosphate (UDP) or guanosine diphosphate (GDP) derivatives are used. According to Ginsburg (49), the use of nucleotides other than UDP and GDP may be necessary for separation of biosynthetic pathways in the cell. Product inhibition and feedback control of sugar nucleotide biosynthesis were recently shown to play a role in bacteria. Bernstein and Robbins (12) found that TDP-glucose pyrophosphorylase is inhibited competitively by UDP-glucose and is inhibited through feedback by TDP-rhamnose in *E. coli* and *Salmonella*. UDP-glucose pyrophosphorylase is inhibited competitively by TDP-glucose and TDP-rhamnose. Melo and Glaser (135) showed that TDP-glucose synthetase is inhibited in *Pseudomonas aeruginosa* by TDP-rhamnose (*see also* 134*b*). Feedback control may account for the finding that TDP-rhamnose is not accumulated in an R mutant of *S. weslaco*, although the enzymes necessary for the synthesis of TDP-rhamnose are present in the mutant cells and rhamnose is not transferred to the lipopolysaccharide (162). On the other hand, TDP-rhamnose was accumulated in an R mutant of *E. coli* O18 (162); here the synthesis of TDP-glucose was not inhibited by TDP-rhamnose (135). Hence, different mechanisms of control are involved in different bacteria. In *S. typhimurium* M mutants, accumulation of TDP-rhamnose and cytidine diphosphate (CDP)-abequose was observed. However, GDP-mannose, which is also a constituent of the wild-type antigen and which is not transferred in the mutant, was not accumulated (150). Kornfeld and Ginsburg (97) have studied feedback control of GDP-mannose and GDP-fucose biosynthesis in several bacteria (*see also* 2*c*, 147*a*).

The defect in R mutants may be due to a block in the activity of either a transferase or one of the sugar-synthesizing enzymes, a synthetase. For a number of mutants, the block has been identified and was shown to be related to one of the enzymes 1 to 6 of Fig. 10. The consequences of the absence of enzyme activity in the bacterial cell for the structure and specificity of the polysaccharide antigens, as well as structural relationships be-

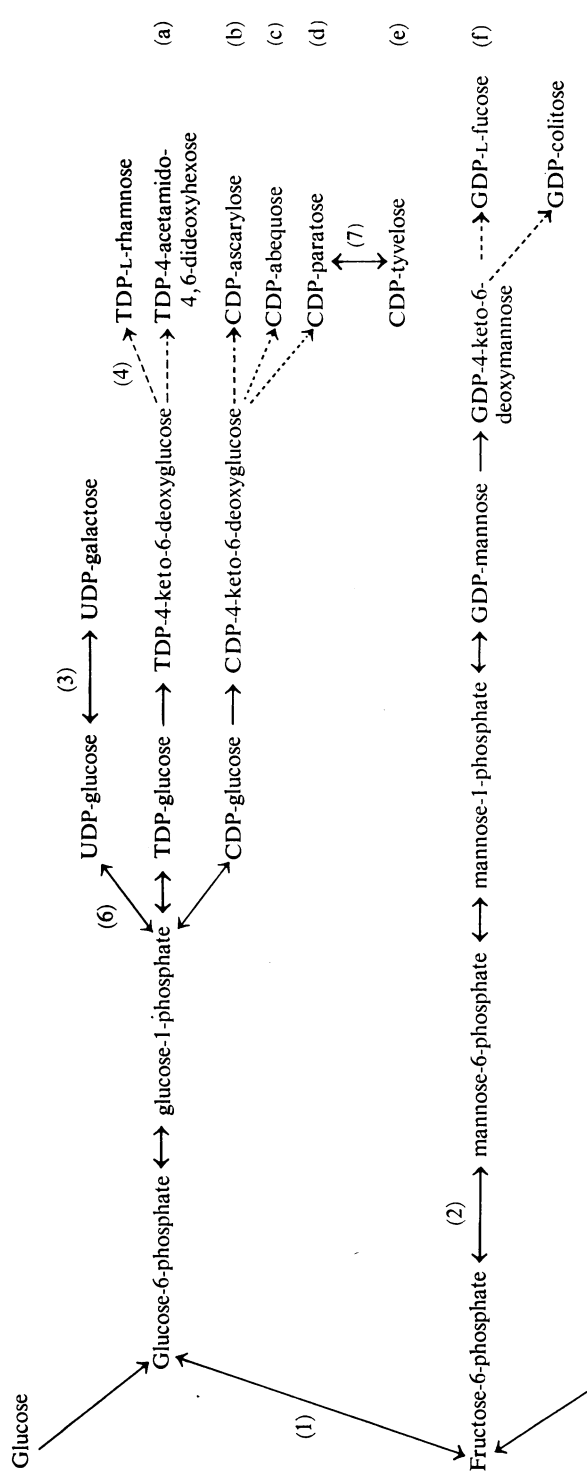


FIG. 10. *Biosynthesis of sugar nucleotides in bacteria [after Ginsburg (49) and Leloir (106)]. Enzymes 1 to 7 are blocked in mutants discussed below: (1) phosphoglucose isomerase, (2) phosphomannose isomerase, (3) UDP-galactose 4-epimerase, (4) TDP-rhamnose synthetase, (5) UDP-N-acetylglucosamine 4-epimerase, (6) UDP-glucose pyrophosphorylase (synthetase) (7) CDP-paratose 2-epimerase. For references see Ginsburg (49) and: (a) Matsuhashi and Strominger (130, 132); (b) Matsuhashi et al. (132), Gabriel and Ashwell (47a); (c) Nikaido (152), Matsuhashi and Strominger (129), Mayer & Ginsburg (134 a, b); (d) Matsuhashi and Strominger (129, 129a); (e) Matsuhashi and Strominger (129), Elbein (33c); (f) Elbein and Heath (33b).*

tween different R antigens and the corresponding O antigens are discussed below.

Preparation and Composition of R (Lipo)polysaccharides

Like the S lipopolysaccharides, R lipopolysaccharides can also be obtained from the corresponding R forms by the phenol-water method (252, 253, *see also* 68*b*). Extraction of the cells with acetic acid (41) yields the degraded R polysaccharides. In contrast, some extraction methods which have been successfully applied to S forms are not suitable for R forms, e.g., the trichloroacetic acid method of Boivin or the diethyleneglycol method of Morgan (24).

The yield of R lipopolysaccharides is generally smaller (less than 1% of the dry weight) than that from corresponding S forms (2 to 3%). R lipopolysaccharides are less soluble in water and may contain more than 50% of material which behaves like lipid A in forming a chloroform-soluble precipitate after hydrolysis with weak acid.

F. Kauffman isolated R mutants from about 25 *Salmonella* S forms belonging to many chemotypes and serogroups. Analyses of the R lipopolysaccharides showed that the specific sugars present in the parent O antigens were absent. All R lipopolysaccharides belonged to chemotype I and contained only the basal sugars KDO, heptosephosphate, galactose, glucose, and glucosamine, regardless of the chemotype of the parent S forms (93, *see also* 252).

Analyses of the degraded R polysaccharides obtained from the R lipopolysaccharides revealed, however, that differences existed with respect to their sugar constituents. In a first group of R lipopolysaccharides, glucosamine represented a constituent of the polysaccharide, and in a second group glucosamine was present only in the lipid portion (122, 123).

Further groups of *Salmonella* mutants have been isolated, in which the antigens were characterized by the absence of further sugars. Nikaido (149, 150) and Heath and Elbein (65) studied mutants of *Salmonella* and *E. coli* whose R polysaccharides lacked glucosamine and galactose. A fourth group of R mutants was described in which the polysaccharides were composed only of KDO and heptosephosphate, lacking glucosamine, glucose, and galactose (40, 45, 123*b*, 232) (Table 14).

Recently, Lüderitz et al. (123*a*) isolated R forms from *S. minnesota* which yielded, on extraction with phenol-water and ultracentrifugation, a substance consisting of lipid A, KDO, and ethanolamine. No hexoses nor heptose could be detected. Similar mutants had been described earlier by Goebel and Jesaitis (54*a*), who obtained a phage-resistant heptoseless variant of phase II *Shigella sonnei*, and by Weidel et al. (245*a*), who analyzed a phage-resistant heptoseless mutant of *E. coli* B.

Chemical analysis thus revealed that the known *Salmonella* R lipopolysaccharides can be classified into five groups according to their sugar composition, each representing a distinct chemotype. The first group of antigens is composed of the five basal sugars, and therefore belongs to chemotype I (*see* 93). R antigens of the second, third, fourth, and fifth group of R mutants contain only 4, 3, 2, or 1 different sugars as constituents of their R polysaccharides, glucosamine being present only in lipid A. These antigens represent new, simpler chemotypes than chemotype I. It is, therefore, proposed to enlarge the table of chemotypes (Table 5) so that the chemotypes of R lipopolysaccharides are designated by letters: chemotype Ra (identical with chemotype I) and chemotypes Rb, Rc, Rd, and Re, respectively (see Table 15) (123*a*). To differentiate between glucosamine as part of lipid A and the glucosamine constituent of the polysaccharide, two symbols have been introduced in Table 15.

In addition to typical R mutants, two groups of mutants have been described which are believed to occupy an intermediate position between S and R types. One includes *Salmonella* strains which were found in nature and which could not be typed by the O sera available. These strains, which grow in smooth colonies and easily undergo mutation to R forms, were designated by Kauffmann as T (transient) forms (86*a*). The second group of intermediate mutants was reported by Naide, Nikaido, Mäkelä, and Stocker (144), who obtained mutants whose antigens contained the basal sugars and, in addition, small amounts of those sugars specific for the parent strains; this class of mutants was called semirough (SR).

R Mutants of Chemotype Ra (Serotype R II Mutants)

Serology. Mutants belonging to this class were first characterized by the serological specificity of their R antigen (10). The lipopolysaccharides extracted from R II mutants cross-react with rabbit antiserum against the test strain of this group (*S. inverness R II*) in hemagglutination and hemagglutination-inhibition tests, and not with antiserum to the test strain of serogroup R I (*S. minnesota* R I). Table 16 shows the serological

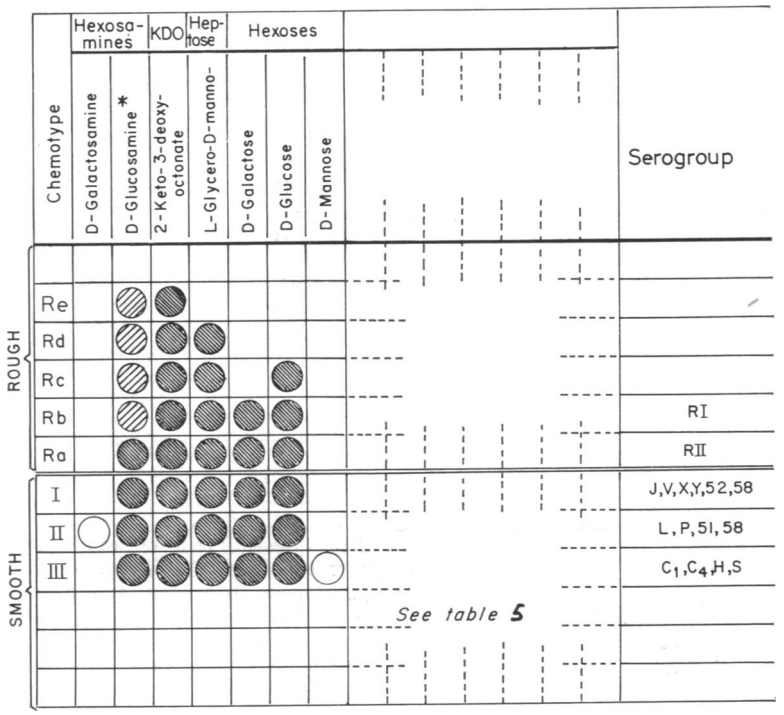
TABLE 14. *Classification of Salmonella R mutants including Escherichia coli K-12 mutant according to sugar constituents of the specific polysaccharides and the enzymatic defect**

Determination	R chemotype a					R chemotype b		R chemotype c		R chemotype d	
	S. minnesota (Kauffmann)†	*S. typhimurium* TV 208 (Stocker)	*S. poona* (Kauffmann)	*S. inverness* (Kauffmann)	*S. typhimurium M 2* (Osborn)	*S. minnesota* (Kauffmann)	*S. typhimurium* (Stocker)	*S. typhimurium M* (Fukasawa)	*S. enteritidis M* (Fukasawa)	*S. typhimurium* mutant (Smith)	*E. coli* K-12 mutant (Lederberg)
Sugar composition of wild-type polysaccharide	GalN	Abe Rha Man	GalN Fuc	GalN	Abe Rha Man	GalN	Abe Rha Man	Abe Rha Man	Tyv Rha Man	Abe Rha Man	
	+BS	+BS	+BS	+BS	+BS	+BS	+BS	+BS	+BS	+BS	+BS
Sugar composition of R mutant polysaccharide	GlcN Gal Glc Hep KDO	GlcN Gal Glc Hep KDO	GlcN Gal Glc Hep KDO	GlcN Gal Glc Hep KDO	GlcN Gal Glc Hep KDO	— Gal Glc Hep KDO	— Gal Glc Hep KDO	— — Glc Hep KDO	— — Glc Hep KDO	— — — Hep KDO	— — — Hep KDO
Serogroup	R II	R II	R II	R II	ND	R I	R I	ND	ND	ND	ND
Enzymatic defect	UDP-GlcNAc epimerase	TDP-Rha synthetase	ND	ND	Phospho-Man Isomerase	Transferase of basal sugars		UDP-Gal-epimerase		Phospho-Glc isomerase	UDP-Glc synthetase

* BS = basal sugars (GlcN, Gal, Glc, Hep, KDO); ND = not determined; Abe = abequose; Fuc = fucose; Gal = galactose; Glc = glucose; Hep = Heptose; Man = mannose; Rha = rhamnose; Tyv = tyvelose; KDO = 2-keto-3-deoxyoctonate; TDP = thymidiine diphosphate; UDP = uridine diphosphate; GalN = galactosamine; GlcN = glucosamine; GlcNAc = N-acetylglucosamine.
† Indicates person who isolated the strain.

classification of R lipopolysaccharides based on hemagglutination-inhibition tests. Of 27 R strains, 21 belonged to serogroup R II. The lipopolysaccharides of the 21 organisms, in concentrations of 0.25 to 0.4 μg/ml, inhibited agglutination of erythrocytes coated with *S. inverness* R II lipopolysaccharide by a specific antiserum. Of 14 *S. typhimurium* R strains isolated by Subbaiah and Stocker (231), 6 belonged to the R II serogroup (11).

TABLE 15. *Salmonella R chemotypes in relation to S-chemotypes (see also Table 5)*



* Light shaded circles: lipid A glucosamine only. Dark shaded circles: lipid A and polysaccharide glucosamine. Open circles: sugar occurring only on the specific side chains.

Chemical structure. Analyses of a number of purified lipid A-free R II polysaccharides showed that glucosamine is not only a constituent of lipid A, but also of the polysaccharide component irrespective of the presence of glucosamine in the O-specific side chain of the parent strain. Table 17 summarizes the results of quantitative analyses of polysaccharides and lipopolysaccharides derived from four different *Salmonella* R II mutants. Although differences in analytical data may occur (123) in different preparations from the same mutant, the molar ratio of the sugars in the polysaccharides is close to two heptoses, two glucoses, two galactoses, and one glucosamine. In about 15 other R II lipopolysaccharides the ratio of galactose to glucose ranged between 1.1 and 1.3.

Evidence as to the structure of R II antigens was obtained from analyses of oligosaccharides derived from partial hydrolysates of *S. minnesota* R II lipopolysaccharide. Figure 11 summarizes the results, as well as the relationship among the six oligosaccharides obtained (233). Four other R II lipopolysaccharides, derived from *S. typhimurium* R II/TV157, *S. typhimurium* R II/TV208, *S. poona* R II, and *S. inverness* R II, were also investigated, the last being the test strain used for serogroup R II classification. From partial hydrolysates, three oligosaccharides were isolated which were identical with oligosaccharides 1, 2, and 4 of Fig. 11. Oligosaccharides 3 and 6 were also isolated from *S. typhimurium* R II/TV208 lipopolysaccharide (233). M. J. Osborn, working with the lipopolysaccharide of a GDP-mannose deficient mutant of *S. typhi-*

TABLE 16. *Serological classification of Salmonella R lipopolysaccharides**

R serogroup	Lipopolysaccharide from serotypes	Serogroup of the S form	Chemotype of the S form	Hemagglutination inhibition	
				System 1† (μg/ml)	System 2† (μg/ml)
I	*S. berlin* R	J	I	8.0	250
	S. bergen R	X	I	1.0	250
	S. minnesota R_1‡	L	II	1.0	250
	S. worthington R	G	VI	2.0	250
	S. monschaui R	O	X	4.0	250
II	*S. typhimurium* R_1‡	B	XIV	>250	2.0
	S. hvittingfoss R	J	XII	>250	4.0
	S. inverness R_1‡	P	II	>250	1.0
	S. typhi R	D	XVI	>250	1.0
	S. minnesota R_2‡	L	II	>250	0.5
	S. binza R	E_2	XIII	>250	0.5
	S. telaviv R	M	IX	>125	0.5
	S. weslaco R	T	VII	>250	0.5
	S. poona R	G	VI	>250	0.5
	S. bareilly R	C_1	III	>250	0.5
	S. paratyphi C R	C_1	III	>125	<0.25
	S. inverness R_2‡	P	II	>250	<0.25
	S. paratyphi A R	A	XV	>250	<0.25
	S. newport R	C_2	XIV	>250	<0.25
	S. aberdeen R	F	XIII	>125	<0.25
	S. greenside R	Z	XI	>125	<0.25
	S. deversoir R	W	V	>250	<0.25
	S. dugbe R	W	V	>250	<0.25
	S. cerro R	K	IV	>250	<0.25
	S. dahlem R	Y	I	>250	<0.25
	S. djakarta R	Y	I	>250	<0.25
	S. typhimurium R_2‡	B	XIV	>250	>250

* Results of hemagglutination inhibition tests [after Beckmann, Lüderitz, and Westphal (10)].

† Hemagglutinating system 1: *S. minnesota* R_1 lipopolysaccharide (250 μg/10 ml of a 0.5% suspension of formaldehyde-treated erythrocytes)/*S. minnesota* R_1 antiserum (three agglutinating units). System 2: *S. inverness* R_1 lipopolysaccharide (250 μg/ml of a 0.5% suspension of formaldehyde-treated erythrocytes)/*S. inverness* R_1 antiserum (three hemagglutinating units).

‡ R_1 and R_2 are R forms of the same species isolated at different times.

TABLE 17. *Analysis of Salmonella R II polysaccharides and lipopolysaccharides**

Prepn†	KDO	Heptose	Glucose	Galactose	Glucosamine
S. minnesota R II					
Freeman polysaccharide	2	16	17	16	5.5
Polysaccharide isolated from lipopolysaccharide	2	21	13	16	5.6
S. typhimurium TV 157 R II					
Lipopolysaccharide		7	5.7	5.9	9.3
S. poona R II					
Lipopolysaccharide		8.4	4.9	5.5	9.3
S. inverness R II					
Lipopolysaccharide		9	5.0	6.2	9.0

* According to Lüderitz et al. (123) and Sutherland et al. (233). Sugar content is expressed as percentage of dry weight of the respective preparation.

† The total percentage of sugar in these preparations is lower than usually found in corresponding preparations derived from S forms.

murium, isolated a similar series of oligosaccharides which are probably identical (*personal communication; see also* 167) with those found in our laboratories.

These results indicate that the five R II polysaccharides thus far examined contain a similar or identical structure composed mainly of heptose-phosphate and oligosaccharide 6 of Fig. 11. The structure proposed in Fig. 12 for chemotype Ra polysaccharides is in agreement with these findings. The details of this formula are based upon additional results with other *Salmonella* mutants and on the biosynthetic studies discussed below. Chains of oligosaccharide 6 are linked to a heptosephosphate backbone in such a way that each second heptose residue carries a side chain. The isolation, in small amounts, of a galactose-heptosephosphate as a split product from the R polysaccharide suggested that some heptose units might carry a galactose residue (233). This structure is assumed to be specific for (lipo)polysaccharides of *Salmonella* R II mutants in general, and to constitute the common core of *Salmonella* O antigens.

	R II	R I
1	α-GlcNAc → glucose	
2	α-glucose → galactose*	α-glucose → galactose
3	glucose (↑6, |1) α-galactose	glucose (↑6, |1) α-galactose
4	α-GlcNAc → α-glucose → galactose	
5	α-GlcNAc → α-glucose → galactose → glucose	
6	α-GlcNAc → α-glucose → galactose → glucose (↑6, |1) α-galactose	
7		α-glucose → galactose → glucose (↑6, |1) α-galactose

FIG. 11 . Oligosaccharides from partial hydrolysates of *Salmonella minnesota R I and R II lipopolysaccharides* [*Sutherland et al.* (*233*)].

* *Neither α-glucose-(1 → 4)-galactose nor α-glucose-(1 → 6)-galactose. GlcNAc = N-acetylglucosamine.*

Enzymatic defects. Insofar as R II mutants have been analyzed biochemically and the specific enzymatic defect has been established, the block has always been related to the synthesis of the specific side chain.

Nikaido et al. (154) have analyzed the defect in the *S. typhimurium* R II mutant TV 208 (Table 14). The lipopolysaccharide of the parent strain contains the basal sugars and additional mannose, rhamnose, and abequose. By comparative analyses of cell extracts of mutant and wild-type cells, the authors found that the mutant cells were unable to convert TDP-glucose into TDP-rhamnose. An intermediate of the reaction, TDP-4-keto-6-deoxy-D-glucose (TDP-KDG) (161) is not transformed by mutant cell extracts into TDP-rhamnose (enzyme 4 in Fig. 10). This TDP-rhamnose deficient mutant showed a relatively high rate of back mutation, in contrast to an *E. coli* K-12 strain (Y 10) having the same block (161).

Similar studies on the R II mutant of *S. minnesota* isolated by Kauffmann (Table 14) revealed that the enzyme UDP-*N*-acetylglucosamine 4-epimerase (enzyme 5 of Fig. 10) was absent. This enzyme catalyzes the synthesis of *N*-acetylgalactosamine, a specific constituent of the wild-type O antigen. The *S. minnesota* R II strain, therefore, is a UDP-acetylgalactosamine deficient mutant (123).

With the aid of phage P22, Osborn et al. (167, 266) isolated a mannose-negative R mutant (Table 14) from a culture of *S. typhimurium* treated with ethyl methanesulfonate. Biochemical analysis showed it to be a GDP-mannose deficient mutant. The mutant cell did not contain phosphomannose isomerase (enzyme 2 of Fig. 10) which interconverts fructose-6-phosphate and mannose-6-phosphate, and therefore is unable to synthesize GDP-mannose when cultivated on glucose. Again, in this mutant the synthesis of a specific sugar, mannose, is blocked. The mutant has not

yet been classified serologically. When it is grown in the presence of exogenous mannose, the enzymatic defect is bypassed and the wild-type lipopolysaccharide is formed. A cell-free extract contains all the enzymes necessary for the contains mannose or rhamnose in the R lipopolysaccharide, although these sugars occupy adjacent positions in the O-specific side chain of the wild-type lipopolysaccharide. In the lipopolysaccharide of the GDP-mannose deficient mutant,

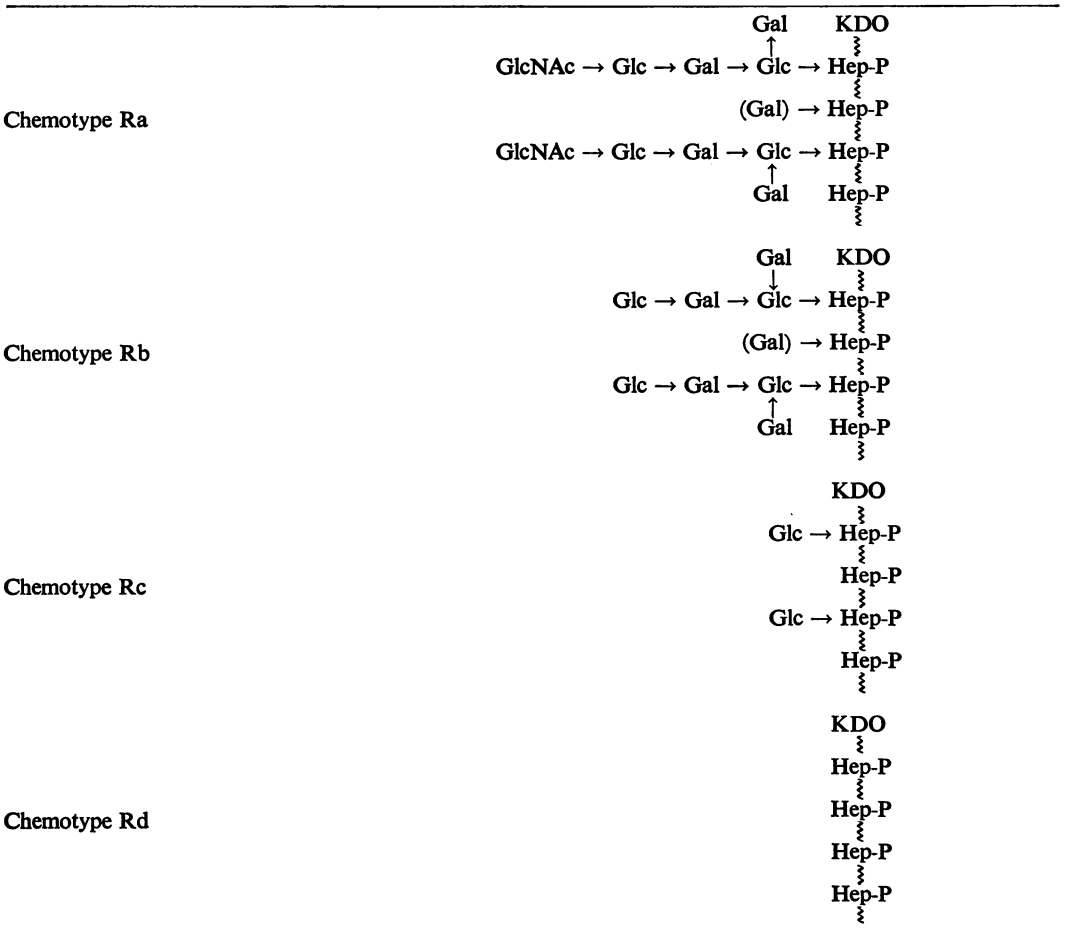


FIG. 12. *Proposed structures of Salmonella R polysaccharides. As to chemotype Rb this is the most completed structure realized, for instance, by S. minnesota R I; chemically more reduced structures occur in members of this chemotype. GlcNAc = N-acetylglucosamine; KDO = 2-keto-3-deoxyoctonate; Glc = glucose; Gal = galactose; Hep-P = heptose phosphate.* For heptose backbone structure see (102*a*).

conversion of mannose into GDP-mannose. As there is no loss in mannose (no conversion to fructose), the yield of GDP-mannose is high, and the system can be used for the preparative synthesis of GDP-mannose (Osborn, *personal communication*) from mannose.

The results demonstrate that in some R II mutants the total absence of the O-specific side chains is caused by a defect in the synthesis of only one specific sugar. Neither the TDP-rhamnose deficient mutant nor the GDP-mannose deficient mutant of *S. typhimurium* however, small amounts of rhamnose were found (about 1 rhamnose per 10 glucosamine units) (167). This could mean that those sugars preceding mannose in the specific side chain of *S. typhimurium* antigen are incorporated to some degree (for instance, galactose and rhamnose).

In other R II mutants, no enzymatic defect could be detected (154). In these instances, the defect may be related to the absence of a transferase which in the parent strain would transfer a specific sugar to the side chain. It is difficult to prove conclusively that a transferase is absent.

In conclusion, phenotype R II can be expressed by different genotypes; i.e., mutants blocked in the synthesis or transfer of one of the sugar constituents of the O-specific main chains synthesize the R II antigen.

R Mutants of Chemotype Rb (Serotype R I Mutants)

Serology. With the aid of an antiserum to the R I mutant of *S. minnesota* (Table 16), a second group of R lipopolysaccharides was identified, namely, the serogroup R I (10). R I lipopolysaccharides do not inhibit the *S. inverness* R II system, even in high concentration, but they do inhibit the *S. minnesota* R I system in concentrations from 1 to 8 μg/ml (Table 16). Mutation to R I specificity, as in the R II mutation, is independent of the chemo- or serotype of the parent strain. Kauffmann and Stocker have isolated R I as well as R II mutants from both *S. minnesota* and *S. typhimurium*.

Chemical structure. R I lipopolysaccharides do not contain glucosamine in the polysaccharide component. Comparative analyses of *S. minnesota* R I lipopolysaccharide and polysaccharide are presented in Table 18. The ratio of galactose to glucose is 1.6 to 2.0. Four other R I lipopolysaccharides in Table 16 gave ratios between 1.7 and 2.0; the ratio was about 1 in the lipopolysaccharide from the *S. typhimurium* Rb/166 mutant isolated by Subbaiah and Stocker (233) (Table 18).

Partial hydrolysates of *S. minnesota* R I lipopolysaccharide yielded three oligosaccharides, two of which were identical with those from *S. minnesota* R II lipopolysaccharide (Fig. 11), indicating that *S. minnesota* R I polysaccharide possesses a structure analogous to that of R II, but lacking the terminal reducing *N*-acetylglucosamine (Fig. 12). From *S. typhimurium* Rb/166, two unidentified disaccharides (α-glucose → galactose) and the disaccharide α-galactose-(1 → 6)-glucose (3 in Fig. 11) were isolated (233).

The varying glucose-galactose ratios as well as the analyses of the partial hydrolysates show that, in contrast to the R II group, the structures of the different Rb polysaccharides are not necessarily identical. *S. typhimurium* Rb/166 polysaccharide seems to possess a simpler structure than *S. minnesota* R I polysaccharide. However, the lipopolysaccharide of Rb/166 cross-reacts with that of *S. minnesota* RI, possibly due to the common α-galactose-(1→6)-glucose residue, as shown by hemagglutination tests (11) as well as by hemagglutination-inhibition of several R I antisera (*unpublished data*).

TABLE 18. *Analysis of Salmonella R I polysaccharides and lipopolysaccharides**

Prepn	KDO	Heptose	Glucose	Galactose	Glucosamine
S. minnesota R I					
Lipopolysaccharide†	5	12	4	8.5	5
	5	12	4	8.5	8
Polysaccharide†‡	2	19	9	19	(1)
	2	15	12	20	(0.8)
S. typhimurium TV166 R I					
Lipopolysaccharide	8	13.7	6.7	6.9	7.3
Polysaccharide	5.7	23.7	13.5	13	(0.6)

* According to Lüderitz et al. (123) and Sutherland et al. (233). Sugar content is expressed as percentage of dry weight of the respective preparation.

† Results obtained from two different preparations.

‡ Obtained by hydrolysis of the lipopolysaccharides.

Enzymatic defects. The defects of R I mutants have not yet been located with certainty. In R I mutants of *S. typhimurium*, the enzymes necessary for the synthesis of the sugars of the wild form were present in amounts comparable to those in the parent strains (154). Also, *S. minnesota* R I mutant contained about the same activity of UDP-*N*-acetylglucosamine epimerase as did the wild strain (123). Since in RII mutants the synthesis of the O-specific side chains is blocked, in R I mutants the block may possibly be linked to the synthesis of the basal core structure. A defect in the synthesis of sugar nucleotide precursors (UDP-acetylglucosamine, UDP-galactose, UDP-glucose) of the basal structure can be excluded, since these sugars and their nucleotide derivatives are found in R I cells. According to biosynthetic studies (see below), there are no present indications that hexose nucleotides other than UDP-derivatives are involved in the synthesis of the basal core polysaccharide. It seems likely, therefore, that in R I mutants a trans-

ferase is lacking which in the wild type transfers a basal sugar into the R I structure, thus converting it into the complete core polysaccharide. As a consequence, the determinant sugars forming the O-specific side chains in the respective O antigen cannot be transferred because the acceptor, i.e., the intact basal core structure, is not synthesized. Since several transferases are necessary for the synthesis of the complete basal structure, it is concievable that R mutants lacking different transferases could occur, so that the structures (completeness) of their R antigens would vary accordingly. Some of these would exert R I specificity, possibly due to the nonreducing terminal galactose in the small side chain of the core polysaccharide. This would be in accordance with the finding that in passive hemagglutination tests serological cross-reactivity can be demonstrated to occur between R II lipopolysaccharides and R I antisera (10). This reaction is not reciprocal and was not observed in hemagglutination-inhibition tests in which the sera are used in higher dilution.

O-specific polysaccharide hapten of R I mutants. For the isolation of lipopolysaccharides, dried bacteria are generally extracted with phenol-water. High-speed centrifugation of the crude extract yields a jelly-like sediment, the lipopolysaccharide, and a supernatant fluid, called L 1 fraction (11, 123), whose major constituent is nucleic acid and which may occasionally also contain homopolysaccharides, such as glucan.

Although the lipopolysaccharides of chemotypes Ra and Rb contain the basal sugars only, paper chromatographic analyses of hydrolysates of L 1 fractions from many *S. typhimurium* R mutants occasionally revealed the unexpected presence of mannose, rhamnose, and abequose (11). These sugars were present in the L 1 fractions derived from R I mutants, but not in the corresponding fractions of R II mutants, and constitute a specific polysaccharide found only in R I cells. The specific polysaccharide of one of the R I-L 1 fractions was separated from contaminating nucleic acid with the aid of Cetavlon. The following monosaccharide constituents were found: hexosamine, galactose, glucose, mannose, rhamnose, abequose, and small amounts of ribose and xylose (I. Beckmann, *unpublished data*). Heptose and phosphorus were not detected. Specific anti-*Salmonella* B serum precipitated the polysaccharide and agglutinated erythrocytes sensitized with it.

The L 1 fraction of *S. minnesota* R I mutant also contained a polysaccharide that could be isolated by specific precipitation with *S. minnesota* O antiserum and which contained galactosamine, the specific sugar of the wild-type antigen (123). The specific polysaccharide in R I cells, however, does not give rise to the formation of anti-O antibodies after immunization of rabbits with the R I bacteria. The specific polysaccharide has the properties of a hapten. [However, R antisera may occasionally contain O agglutinins or O hemagglutinins, if the R cultures used for immunization contain wild-form cells due to revertants, or if R and parent O antigens share a common specificity (120, 123*a*). Cross-reaction is also observed if the mutant belongs to the recently discovered class of SR strains (144)].

R I cells, then, synthesize two different polysaccharides: the R I specific component of the cell wall lipopolysaccharide, and the O-specific polysaccharide hapten. R I mutants have an enzymatic defect involving synthesis of the basal structure. Synthesis of O-specific side chains is not inhibited, but their attachment to the core appears not to be possible because the specific receptor, the complete R II structure, is not fully formed in the R I mutant.

The localization of the O-specific polysaccharide hapten in the R I bacterial cell has not yet been determined, since only whole cells have been extracted. Recently, Milner *et al.* (137) and Anacker *et al.* (2) isolated a polysaccharide hapten from *E. coli* S forms. These authors extracted the cell wall and the protoplasm separately with trichloroacetic acid, and obtained the lipopolysaccharide and polysaccharide, respectively. This polysaccharide hapten behaved in gel precipitation tests and on ultracentrifugation similarly to the Freeman polysaccharide extracted from whole cells. We do not know whether the polysaccharide isolated from R I mutants has any relationship to that found by Ribi and co-workers in S strains. Further studies of the different polysaccharides will be needed to provide an insight into the mechanisms of their biosynthesis. Since O-specific polysaccharide haptens isolated from S or R I forms do not contain heptose, the intact basal core of O antigens is lacking.

R Mutants of Chemotype Rc (M Mutants)

Enzymatic defect and biochemical properties. Chemotype Rc antigens occur in the group of R mutants discovered by Murase (143) and designated "mutabile-type," M mutant (the designation M for these *E. coli* mutants does not mean mucoid). M mutants from *S. enteritidis* and *S. typhimurium* were isolated, and their properties were studied (43, 148, 149, 150, 152, *see also* 33*a*). These mutants lacked UDP-galactose 4-epimerase which interconverts UDP-glucose and UDP-galactose (enzyme 3, Fig. 10 and 13). The inability of the UDP-galactose deficient mutants to produce the epimerase, which is of prime impor-

tance for the catabolism *and* anabolism of galactose, leads to profound changes in biochemical and bacteriological properties compared with the wild form as reviewed by Kalckar (83*a*).

On agar, M mutants form rough colonies. They are nonfermenters of galactose. In the absence of exogenous galactose, the mutant is unable to synthesize galactose. The cell wall lipopolysaccharide, therefore, does not contain galactose and is composed of glucose and heptosephosphate only (glucosamine being present in the lipid). M mutants are galactose-sensitive; when galactose is added to the culture medium, M cells lyse or form protoplasts if the medium is hypertonic. M mutants from *S. typhimurium*, in contrast to the parent strains, are resistant to the action of phage P22. However, within 20 min after addition of a small amount of galactose, just before lysis would occur, wild-type cells are formed which are sensitive to phage P22. In the presence of galactose, UDP-galactose is formed (see Fig. 13), and the O antigen responsible for phage sensitivity can be synthesized (44, 148, 149, 150, 152; *see also* 83). Under special conditions, the amount of UDP-galactose accumulated in the presence of galactose is such that this system can be used for the preparative synthesis of UDP-galactose (142, 263).

Chemical analysis of chemotype Rc lipopolysaccharides. Nikaido (149, 150) has isolated the M lipopolysaccharide from *S. enteritidis* and *S. typhimurium* M mutants. Glucose and heptose were the only constituents of the polysaccharide component. M mutants, cultivated in the presence of galactose, yielded a wild-type lipopolysaccharide containing all of the O-specific sugars.

Besides heptose and glucose, M lipopolysaccharides contain glucosamine as one of the constituents of lipid A, 2-keto-3-deoxyoctonate (KDO), and ethanolamine.

Since its discovery by Heath and Ghalambor (66), KDO has been found to occur in all lipopolysaccharides studied so far. In an extended study on the role of KDO in lipopolysaccharides, Osborn (164) showed that KDO occurred in two different linkages. About 75% is bound terminally in a nonreducing glycosidic linkage which is acid-labile and alkali-stable. When the lipopolysaccharide is split by weak acid, the rest (about 25%) of the KDO is found in the polysaccharide part. In the polysaccharide, KDO could be determined by the thiobarbituric acid reaction (244, 247) without prior liberation. The position of the KDO residues in the degraded polysaccharide is terminal but reducing, and the linkage is acid-stable. Osborn suggested that terminal KDO of the polysaccharide forms the linkage to lipid A in lipopolysaccharides, an assumption which agrees with the fact that the glycosidic linkage of KDO in the lipopolysaccharide is split at about the same rate as lipid A is split off.

The degraded M polysaccharide is said (164) to have a molecular weight of about 4,000 to 5,000. Quantitative analysis of Sephadex-separated fractions of degraded M polysaccharide showed a molar ratio of heptose to glucose to KDO of approximately 10:5:1 (Table 19).

Osborn proposed the structure of M polysaccharides shown in Fig. 12 (chemotype c) on the basis of chemical and analytical studies in terms of the relationship to other R lipopolysaccharides, as well as on the basis of biosynthetic data. This structure represents the innermost part of the core of O antigens, polyheptosephosphate (the backbone) with additional glucose. At present, nothing is known of the nature of the linkage between glucose and heptose, or the linkage between the heptosephosphate units. Recently, a glucosyl-heptose disaccharide was isolated by Nikaido from the lipopolysaccharide of an epimeraseless mutant of *S. enteritidis* (102*a*, 151).

TABLE 19. *Analysis of Salmonella typhimurium M mutant polysaccharide (164)*

M-polysaccharide*	Molar ratio (based on phosphate = 1)			
	Phosphate	Heptose	Glucose	KDO
III	1	1.3	0.65	0.13
V	1	1.1	0.52	0.08

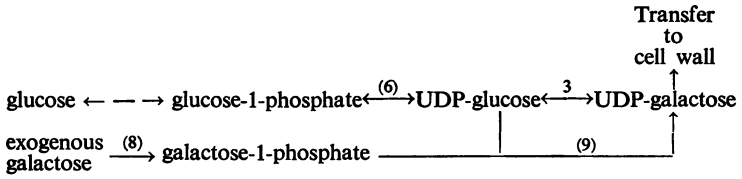
* Diethylaminoethyl cellulose fractions.

Accumulation of nucleoside diphospho sugars in M mutants. R I and M mutants have in common the inability to synthesize a complete basal core structure, but the defect differs in each group. R I mutants probably lack a transferase, but the enzymes necessary for the synthesis of the specific side chains are present and active, as in the wild form. Therefore, R I mutants accumulate the O-specific polysaccharide hapten. In contrast, the defect of *S. typhimurium* and *S. enteritidis* M mutants lies in the synthesis of a sugar, galactose, which is present in the basal core structure as well as in the specific side chain of the corresponding wild type. Accumulation of the O-specific hapten, therefore, is not anticipated in these M mutants and does not occur. On the other hand, analysis of an 80% ethyl alcoholic extract of M mutant cells revealed the presence of appreciable amounts of sugar nucleotides (149, 150). Chromatographic purification of the extract led to the isolation of TDP-rhamnose and of two new sugar nucleotides: CDP-tyvelose from

S. enteritidis M mutant and CDP-abequose from *S. typhimurium* M mutant. Later, Heath and Elbein (33*b*, 65) isolated GDP-colitose from an *E. coli* O111 M mutant (33*a*), and showed that a cell-free extract catalyses the synthesis of GDP-colitose from GDP-mannose (see Fig. 10).

The sugars accumulated in M cells as nucleotide derivatives cannot be transferred to the glucose-heptose core. Neither can they be utilized to build up the O-specific hapten with *Salmonella* group B or D specificity, because UDP-galactose is absent. However, as soon as galactose is added to the culture medium, UDP-galactose is formed (44, 148), the basal structure is completed, and the additional wild-type sugars are transferred. Phenotypically, these cells become S-form cells.

It is probable that M mutants derived from *Salmonella* species lacking galactose as a constituent of the specific side chain (like *Salmonella* group N antigens) accumulate the specific hapten in analogy to R I mutants. One might also expect the existence of transferaseless mutants of chemotype b which accumulate O-specific hapten (*see also 123a*).



(3) UDP-galactose 4-epimerase
(6) UDP-glucose pyrophosphorylase
(8) galactokinase
(9) galactose-1-phosphate uridyltransferase

FIG. 13. *Metabolism of galactose in bacteria (see also Fig. 10).*

R Mutants of Chemotype Rd

Fukasawa, Jokura, and Kurahashi (45) and Sundararajan, Rapin, and Kalckar (232) described a number of *E. coli* mutants which have in common a block in the synthesis of UDP-glucose. These lack the enzyme UDP-glucose pyrophosphorylase, which catalyzes the formation of UDP-glucose from glucose-1-phosphate (enzyme 6 in Fig. 10 and 13). These UDP-glucose deficient mutants are unable to synthesize activated glucose and other sugars, the biosynthesis of which requires UDP-glucose as precursor. Glycogen synthesis in bacteria was shown to occur generally via transglucosylation from adenine diphosphate (ADP)-glucose to a glycogen primer (56). UDP-glucose deficient mutants, therefore, can synthesize glycogen (195, *see also* 133). (The phosphoglucose isomeraseless mutant, on the other hand, can be expected not to synthesize glycogen when grown on fructose.)

The cell walls and lipopolysaccharides of these *E. coli* mutants contained heptose, KDO, and glucosamine; the corresponding parent strains, in addition, contained glucose and galactose.

Kuriki and Kurahashi (102*a*) recently analyzed the degraded polysaccharide from an *E. coli* Rd mutant. They were able to separate by chromatography on paper a uniform phosphorus-free fraction consisting of heptose and KDO in a molar ratio of 10:1. Borohydride reduction and periodate oxidation of the product revealed that KDO occupied a terminal, reducing position and that the heptose units were linked glycosidically probably in 1–2 position. Another fraction containing heptose and phosphorus was also isolated. These results are in agreement with similar findings by Osborn (*personal communication*).

Smith (202) isolated a glucose- and galactose-negative R mutant from *S. typhimurium*, which was identified by Fraenkel, Osborn, Horecker, and Smith (39, 40) as a UDP-glucose deficient mutant, blocked in the enzyme phosphoglucose isomerase (enzyme 1 in Fig. 10). Grown on fructose or gluconate in the absence of exogenous glucose, the cells are unable to synthesize glucose-6-phosphate. UDP-glucose and the sugars normally derived from UDP-glucose are not produced by this mutant. Its cell wall polysaccharide contains heptose, KDO, ethanolamine, and phosphate. In the presence of glucose, the block is bypassed, and a polysaccharide is formed which is indistinguishable from that of the wild type; it contains KDO, galactose, glucose, glucosamine, mannose, rhamnose, and abequose.

Stocker (*personal communication*) has isolated a new *S. typhimurium* strain which was shown by Osborn (*personal communication*) to lack the enzyme UDP-glucose transferase I (see Fig. 16). This strain is phenotypically identical with the UDP-glucose deficient mutants, but is distinct in that it contains the O-specific polysaccharide hapten found earlier in R I mutants.

R Mutants of Chemotype Re

Mutants of this class derived from *Shigella sonnei* and *E. coli* B were described by Goebel and Jesaitis in 1952 (54*a*) and by Weidel et al. in 1954 (245*a*), respectively. They were selected as phage resistant mutants. Recently, Lüderitz et al. (123*a*) obtained about 30 mutants from *S. minnesota* belonging to chemotypes Ra to Re. Chemotype Re mutants synthesize lipopolysaccharides consisting mainly of lipid A (about 70%) and KDO (about 17%). Ethanolamine is also present. Hexoses and heptoses are absent. It is possible, however, that a further unknown constituent takes part in the structure. In hemagglutination-inhibition tests, an antiserum against a heptoseless mutant was shown to be specific for this class. (Correspondingly, antisera obtained with chemotype Rd mutants were specific for heptose containing *Salmonella* lipopolysaccharides when tested under the same conditions.) Chemotype Re mutants exhibit a phage pattern characteristic for this group. Stocker has isolated a heptoseless mutant from *S. typhimurium* which maps at a locus different from rouA (see below; *personal communication*).

Salmonella T Forms

In 1956, Kauffmann (86*a*, 88) described a further class of *Salmonella* mutants designated by him as T (transient) forms because of their intermediate properties between S and R strains. T forms on agar form smooth colonies but are devoid of O specificity and show a high rate of mutation to R forms. They exhibit T specificity. Two serologically distinct groups of T mutants were described by Kauffmann: T 1 forms derived from *S. paratyphi B*, *S. anatum*, *S. friedenau*, and others (*see also* 189, 190), and T 2 forms derived only from *S. bareilly*. T antigens were shown to contain the basal sugars; T 1 antigens contain additional ribose (about 20%) and have a high content of galactose (20%) (123*a*). In hemagglutination-inhibition tests, T 1 and R II lipopolysaccharides exhibit strong cross-reactions; however, T 1 lipopopysaccharides do not cross-react with the ribose-containing lipopolysaccharides of *Salmonella* groups 28_1, 28_2, *52*, and *56* (95, 123*a*, 261*a*). In recombination studies, Sarvas and Mäkelä (187*a*) succeeded in the production of *Salmonella* forms whose lipopolysaccharides carried both T1 and O specificities.

Semirough (SR) Forms

Recently, a new class of *Salmonella* strains was discovered, having properties intermediate between S and R II forms and designated as SR (144).

These SR strains were mutants from *S. typhimurium* or recombinants from interspecific crosses with *S. abony* (*4*, *5*, *12*) as donor and *S. montevideo* (*6*, *7*) as recipient. The SR recombinants did not show any of the O-antigenic specificity of the female parent (*S. montevideo*). SR strains are not susceptible to phage P22. They grow on agar as smooth colonies. In fluid medium, they form a deposit and a turbid supernatant fluid. Serologically, they also behave as intermediates: they are agglutinated in anti-*4* serum, but less so than the corresponding wild strains. They also stimulate the formation of anti-*4* antibodies. SR lipopolysaccharide is precipitated by anti-*4* sera.

Comparative paper chromatography of hydrolysates of the lipopolysaccharides of SR and the corresponding S forms showed that both contained the same sugars, but the spots of mannose, rhamnose, and abequose were much weaker in the SR chromatograms. These results were confirmed by quantitative analysis, and indicate that every second heptose in the lipopolysaccharide of the wild type carries a chain containing about four rhamnoses, whereas in the SR lipopolysaccharide every second heptose has a chain with only about one rhamnose unit.

Naide et al. (144) proposed the schematic formula given in Fig. 14 for SR polysaccharides, in which the determinant chain is composed of only one repeating unit. Results of partial acid hydrolysis of SR polysaccharide also agree with this hypothesis: although mannose→rhamnose and rhamnose→galactose disaccharides were found, no traces of galactose→mannose→rhamnose trisaccharide, which constitutes the major product of partial acid hydrolysis of the S polysaccharide, was produced (155*a*).

The nature of the enzymatic defect of SR strains is not known. As a working hypothesis, the authors assumed (144) that transferase I, which transfers the first sugar of the O-specific side chain to the terminal acetylglucosamine unit of the R II structure, might be different from transferase II, which transfers the first sugar of the second repeating unit (see Fig. 14). A similar argument applies even if the S-specific side chain is built by addition of oligosaccharide repeating units instead of monosaccharides, for one enzyme will be required for attachment of the most proximal unit to *N*-acetylglucosamine, and presumably a different enzyme for the addition of further repeating units to that first attached. Absence of the transferase II would then result in an SR mutant. In several cases of lysogenic conversion (ζ27, ϵ15) the enzymatic change is related to the enzyme analogous to transferase II of Fig. 14 (elongation enzyme). It can be assumed, there-

fore, that the first repeating unit of the O specific chain is unaltered in the converted strain. A mutant which lacks transferase I would be a R II mutant. It would be of interest to know whether such a special R II mutant contains the O-specific hapten.

The same authors (144) have identified a second class of intermediate strains in which the antigen is probably composed of the R II structure carrying a few long O-specific chains, thus leaving free many terminal unsubstituted acetyl-

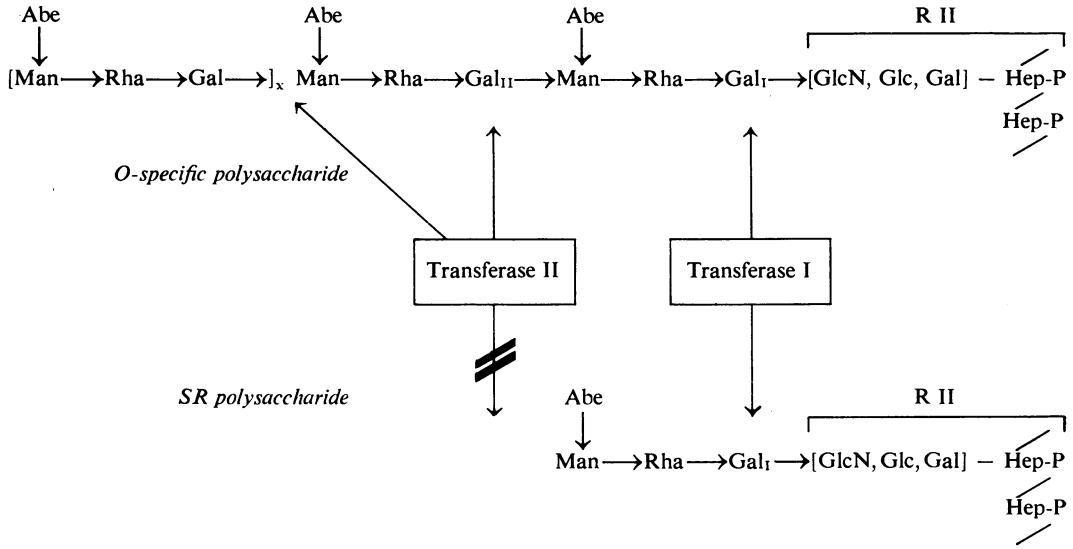


FIG. 14. *Possible structures of the specific polysaccharide of Salmonella S and semirough strains [modified after Naide et al. (144)]. The glucose in the side chain is omitted. If S-specific side chains are synthesized by the sequential transfer of monosaccharide units, transferases I and II would be responsible for the transfer of galactose on to N-acetylglucosamine and to mannose, respectively. If side chains are synthesized by the transfer of oligosaccharide units as such, transferases I and II would function by adding the oligosaccharide to N-acetylglucosamine and to the more proximal repeating unit, respectively. Abe = abequose; Man = mannose; Rha = rhamnose; Gal = galactose; GlcN = glucosamine; Glc = glucose; Hep-P = heptose phosphate.*

glucosamine residues. These strains are susceptible to a host-range mutant of P22 and exert O and R II specificities. Rische et al. (175) have investigated three strains of *S. paratyphi* B isolated from a chronic carrier. Two strains exhibited S and R properties, respectively. One strain was found to be intermediate (I) with respect to quantitative sugar analysis of the lipopolysaccharide (low content of abequose) and to the phage pattern.

Genetics of R Mutants

Subbaiah and Stocker (231) isolated a number of rough mutants from *S. typhimurium* LT 2, a strain in which genetic mapping was feasible through analysis of recombinants obtained by colicine-factor mediated conjugation. Two lines with different markers were used; all the loci concerned had been mapped in a singular circular linkage group. Eighteen R mutants were isolated and studied genetically by crossing each with an S form of the other differently marked line. As a result of these studies, the locus of mutation in 12 R mutants was located at rouA, whereas for 6 other R mutants a different locus was found to be involved, rouB (Fig. 15).

The two classes of genetically differentiated mutants produced antigens of different specificity: five of seven strains mutated at rouA belonged to serogroup R I; six mutated at rouB belonged to serogroup R II (11). From these findings, it can be concluded that locus rouA is concerned in the synthesis of the basal core structure and rouB in that of the O-specific side chains, in agreement with the finding that the loci for factors *5* and *4*, *7* and *9* are near rouB (78*a*, 127, 127*a*). Accordingly, the locus rouA may be alike in many *Salmonella* species, while rouB would be specific for each O group. Stocker et al. (231) crossed a rouA mutant with a rouB mutant in *S. typhimurium*. Besides the expected S form (*rouA*$^+$ *rouB*$^+$) recombinants, *rouA*$^-$ *rouB*$^-$ recombinants were obtained. One such recombinant proved to be R I serologically (11); unlike other R I forms, however, the *rouA rouB* recombinant did not contain O-specific polysaccharide hapten in its L 1 fraction. This was not surprising, as such a

double mutant is blocked both in the synthesis of the basal structure (by the *rouA* lesion) and of the specific side chains (by the *rouB* lesion). A gene, SR (Fig. 15), determining the enzyme transferase II of Fig. 14 was mapped near the gal locus, apart from rouA and rouB (127*a*).

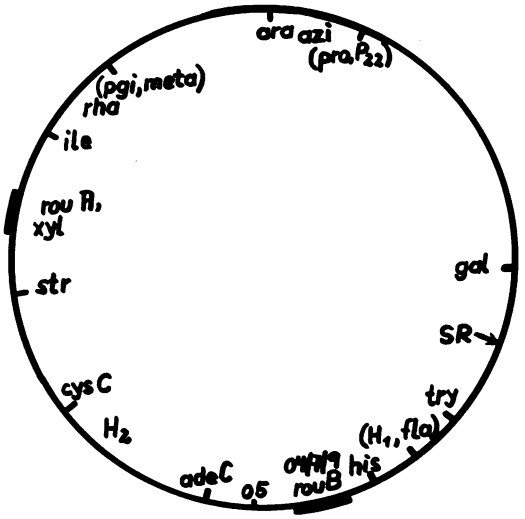


FIG. 15. *Salmonella typhimurium LT2 chromosome [adapted from Subbaiah and Stocker (231), Sanderson and Demerec (186a), and Mäkelä (127, 127a)].*

BIOSYNTHESIS OF *Salmonella* SPECIFIC POLYSACCHARIDES

The structural relationships of different R antigens provide an insight into possible pathways for the biosynthesis of O antigens which, according to the findings, involve the following steps:

Chemotype d antigen →	Chemotype c antigen →	Chemotype b (RI) → antigens
Chemotype a (RII) → antigen	Semirough antigens →	O antigens

In a recent review, Osborn et al. (167) summarized their results on the in vitro biosynthesis of *Salmonella* R and O polysaccharides (*see also* 2*c*, 147*a*). Therefore, only a short summary is presented here.

Nikaido (150) first demonstrated an enzyme (transferase) in cell-free extracts of *Salmonella* M mutants which transfers galactose from UDP-galactose-C^{14} into M lipopolysaccharide. In these experiments, a sonic extract of a *S. enteritidis* M mutant was incubated with UDP-galactose-C^{14}. The extract contained both the enzyme, UDP-galactose transferase, and the aceptor, the galactose-deficient M cell wall (lipopolysaccharide). Appreciable radioactivity was incorporated into the lipopolysaccharide which was isolated by phenol-water extraction. Nikaido thus proved that galactose had been transferred to M lipopolysaccharide (Fig. 16A).

Osborn and co-workers (165, 166, 182, 184) studied the transfer reactions shown in Fig. 16A. Starting with the backbone polysaccharide of the UDP-glucose deficient mutant of *S. typhimurium*, a product was synthesized containing the hexose sequence of the R II structure, with acetylglucosamine as the end group. The side chain shown in Fig. 16A differs from the proposed R II structure of Fig. 12 in that the branching galactose is lacking. Recently, it was shown that galactose is transferred into two different positions of glucoseI (Osborn, *personal communication*).

A sonic extract of the UDP-glucose deficient mutant or the UDP-galactose deficient mutant was usually prepared and a particulate fraction was isolated by differential centrifugation. This fraction contained fragments of the cell wall (lipopolysaccharide) and cell membrane (enzyme). Characterization of the product was achieved by precipitation of the cell wall material with trichloroacetic acid. The resulting precipitate was extracted with phenol-water, and the lipopolysaccharide from the water phase was further purified by precipitation as magnesium salt. Finally, the degraded polysaccharide was isolated after mild acid hydrolysis. This fraction contained up to 80% of the radioactivity of the trichloroacetic acid extract, when the appropriate UDP-C^{14} sugar had been used for incorporation. All transfers were shown to be strictly dependent on the presence of the preceding sugar of the chain. The UDP residue could not be replaced by another nucleotide. It was shown that one of the galactose residues is transferred from UDP-galactose to carbon 3 of the glucose (167).

Rothfield, Osborn, and Horecker (184) succeeded in separating enzyme and acceptor activity. The 105,000 × *g* supernatant fraction of a cell sonic extract contained soluble enzyme but was devoid of acceptor activity. Lipopolysaccharide as such could not function as acceptor. Rothfield and Horecker (183) showed that lipid material, extracted from the particulate cell envelope fraction by chloroform ethanol, could act as a cofactor. By complexing it with lipopolysaccharide, an active acceptor for the enzymatic incorporation of sugars was formed (184*a*). The active component of the lipid was identified as phosphatidyl ethanolamine, and therefore is different from lipid A. It is assumed that the lipid provides an essential site for enzyme binding or alters the physical state of the lipopolysaccharide.

The results described show that the core polysaccharide of *Salmonella* O antigens is synthesized by sequential addition of monosaccharides to the

growing polysaccharide. However, in the synthesis of the O-specific side chains, consisting of repeating units of oligosaccharides, other mechanisms seem to be involved. Nikaido and Nikaido (151, 153, 155) showed that it was possible to incorporate rhamnose into a fraction containing the cell wall polysaccharide of a *S. typhimurium* TDP-cific main side chain is influenced by the presence of other sugars (155, 178, 267). In any case, major differences exist in the mechanism as compared with the synthesis of R polysaccharides. Very recently Osborn and Robbins independently found (*246a*, *265a*; *see also* *2a*, *2b*, *132a*) that the initial reaction in biosynthesis of the O-specific chain is a

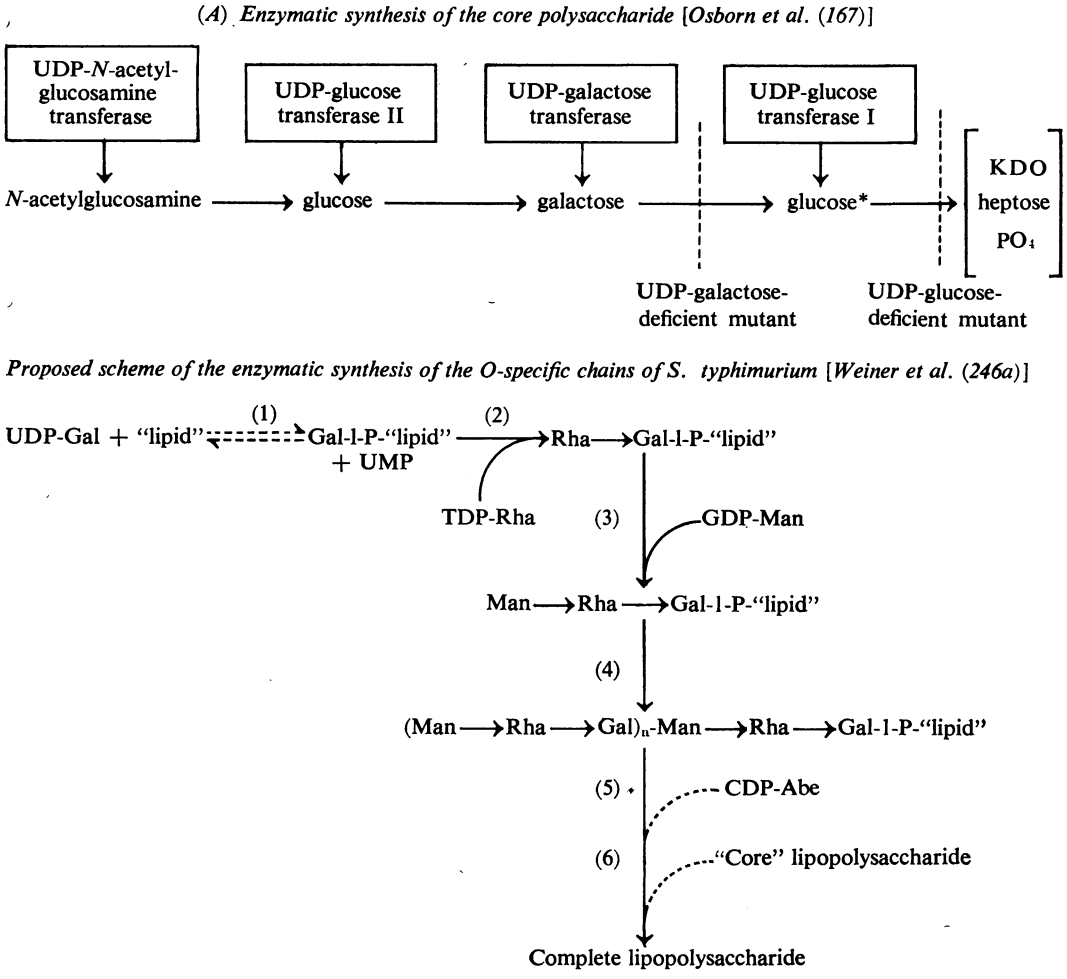


FIG. 16. *Enzymatic synthesis of O-specific polysaccharides of Salmonella.*

* The nonreducing galactose side chain attached to glucose is omitted.

rhamnose deficient mutant. Zeleznick et al. (267) were able to incorporate mannose into the cell wall polysaccharide of the *S. typhimurium* GDP-mannose deficient mutant. In both cases, incorporation of the sugar was markedly stimulated by the presence of the nucleotides of the three sugars present in the repeating unit which forms the O-specific main chain of the wild-type antigen, i.e., UDP-galactose, GDP-mannose, and TDP-rhamnose. The incorporation of one sugar into the specific transfer of sugars to a lipid fraction, leading to a polysaccharide-1-phosphate-lipid intermediate from which the polysaccharide probably is transferred to the "core" lipopolysaccharide. The different steps proposed for the biosynthesis of the O-specific chains are demonstrated on Table 16B. As shown by Robbins (*personal communication*), small side chains (Glucose in factor *34*) and also the acetyl group of factor *10* are transferred after the main chain is synthesized (179, *see also* 241*a*).

Edstrom and Heath (32, 134) studied the M mutant of *E. coli* O111:B4, whose wild-form O antigen contains the basal sugars and colitose. With enzyme and acceptor preparations analogous to those used by Nikaido and Osborn, the existence was demonstrated of analogous lipopolysaccharide transferases which realized the successive incorporation of galactose, glucose, and acetylglucosamine into the M polysaccharide of the *E. coli* mutant. In a subsequent transferase reaction, they could transfer colitose from GDP-colitose as the next sugar. Although the sequence of sugars constituting the core of *E. coli* O111 is the same as in *Salmonella*, the linkages of the sugars appear to be different. A disaccharide isolated both from the enzymatically prepared material and from mild hydrolysates of *E. coli* O111 polysaccharide (32*a*, *see also* 133*a*) proved to be α-glucose (1–4)-galactose which is distinct from the analogous disaccharide isolated from *Salmonella* R II antigen (2 in Fig. 11). Furthermore, the glucosaminyl residue in *E. coli* O111 antigen is β-linked, in contrast to the α-linked glucosamine in *Salmonella* R II polysaccharide. Differences in the basal structure of the antigens derived from *Salmonella* group O35 and *E. coli* O111 had been predicted; although the two organisms show strong cross-reactions (see Table 7), degradation products obtained by mild hydrolysis of the two antigens are serologically distinct (120).

Edstrom and Heath (32) studied the incorporation of KDO into lipopolysaccharide. As with acetylneuraminic acid, KDO is activated through its monophosphate nucleoside derivative, cytidine monophosphate (CMP)-KDO (48), and not through the diphosphate nucleoside, as are the hexoses. KDO could be transferred from CMP-KDO, by means of the particulate enzyme fraction from *E. coli* O111 cells, to a preparation obtained from *E. coli* O111 lipopolysaccharide by partial degradation first with alkali then with acid. This fraction represents a partially degraded lipid A, composed of glucosamine, β-hydroxymyristic acid, and phosphorus. The lipopolysaccharide itself was not an acceptor. In the synthetic product, KDO was linked as a glycoside; this conclusion was based on its alkali stability and acid lability, and on the fact that the carbonyl group could be reduced with $NaBH_4$ only when the product was first treated with acid. The results are in agreement with the concept of Osborn (164) that KDO plays a role as a link between polysaccharide and lipid A in lipopolysaccharides.

General Discussion and Conclusions

This section of the review summarizes the conclusions which have been reached concerning the general structure of the O and R polysaccharides and outlines important and as yet unsolved problems, both old and new. Currently available knowledge on the biology, biosynthesis, and immunology of the polysaccharides is also discussed. Finally, the potential use of the new findings for a genetic classification of *Salmonella* and other *Enterobacteriaceae* is considered.

Chemical Constitution of the O and R Polysaccharides

As shown schematically in Fig. 1, the O polysaccharide, in accordance with the working hypothesis proposed by Kauffmann et al. (93, 117), contains a central core, common to S and many R *Salmonella*. In S forms, the long side chains carrying the specific O factors are attached to this core.

Advances in our knowledge of the constitution of the side chains emerged from immunochemical studies on S forms. In hydrolysates of polysaccharides from groups B, D_1, E, G, N, and U, a limited number of oligosaccharides could be detected in reasonable amounts. Each could be correlated with one oligosaccharide, which therefore is considered to be the repeating unit of the side chains of the respective antigen. Dimers and trimers of such units have actually been isolated and analyzed in groups E_1, E_2, (176, 177), and B(98). It is thus possible to propose for the structure of the side chains of groups B, D_1, and E a sequence of trisaccharide units, namely, galactose→mannose→rhamnose. Secondary side chains of 3,6-dideoxyhexose (on mannose) or glucose (on galactose), or both (see Table 8), may be attached to these trisaccharides.

In the case of groups G, N, and U antigens it is not yet known how the chemically isolated units (see Table 8) are bound together to form the O-specific side chains. For the group U polysaccharide, we assume that the units are linked so that the galactose→galactose disaccharide forms branches on the main chain, an assumption which would explain the blood group B specificity of the U antigen (196).

The tri-, tetra-, or pentasaccharides isolated from partial hydrolysates of O antigens are "chemical units," i.e., chemically isolated units which need not be identical with the "biological repeating units" building up the O-specific side chains. The liberation of the chemical units during acid hydrolysis is dependent upon the relative acid lability of the glycosidic linkages of the sugars constituting the polysaccharide chains, e.g., rhamnose in polysaccharides of *Salmonella* groups B, D_1, or E, or fucose in groups G, N, or U. In group B, the specific side chain most probably begins with galactose, followed by

rhamnose, then mannose, again galactose, and so on (155). The biological repeating unit would then be based on a trisaccharide with the structure, mannose → rhamnose → galactose (155*a*).

The chemical structure of the core polysaccharide was deduced from studies on the R antigens. It was found that different groups of R antigens exist which do not belong to one and the same chemotype, i.e., they do not possess the same key biosynthetic intermediate as the common basal polysaccharide core of O-specific *Salmonella* polysaccharides. Its nonreducing *N*-acetyl-D-glucosamine unit (Fig. 17) might form the link to the repeating units of the long O-specific side chains. This concept is based upon the following findings.

(i) All *Salmonella* O antigens contain the same five basal sugars, KDO, glucosamine, heptose,

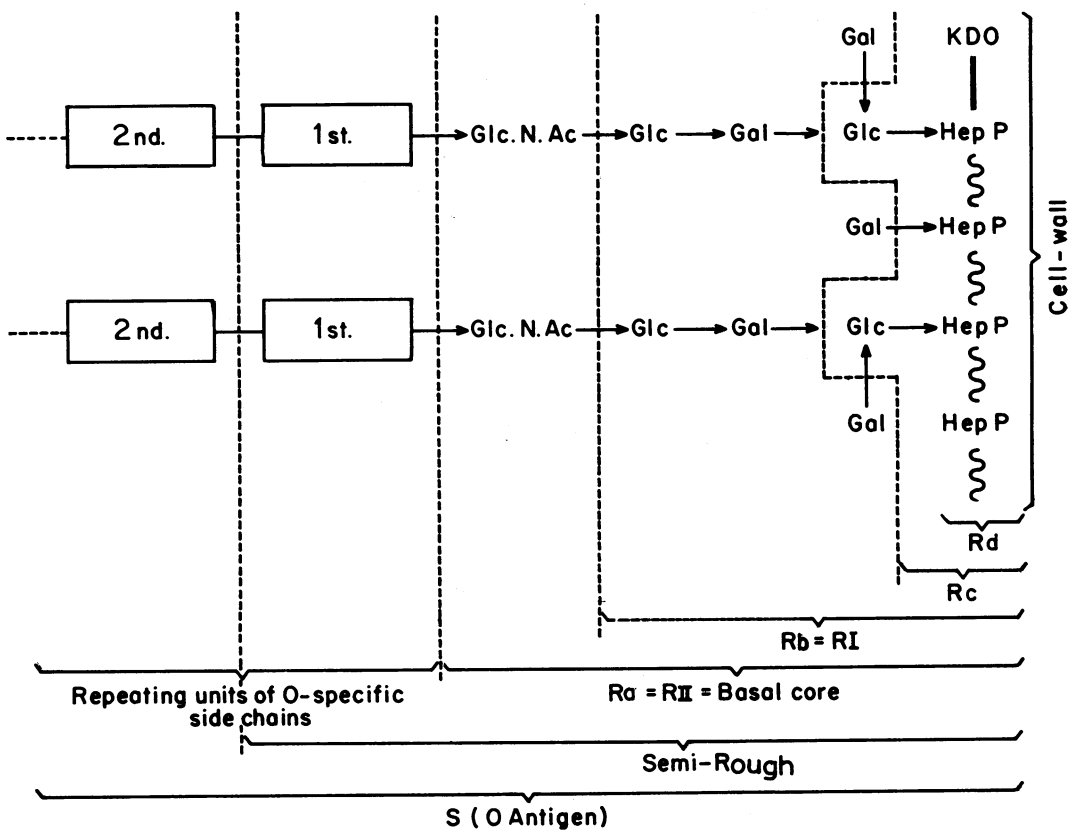


FIG. 17. *Possible structure of Salmonella O and R antigens. (See also legend of Fig. 12.)*

sugar composition. There are at least five different chemotypes Ra, Rb, Rc, Rd, Re. Chemical analyses as well as biosynthetic studies suggest that the chemotypes Rb, Rc, Rd, and Re are simpler forms than chemotype Ra. The latter would consist mainly of a central chain of heptose-phosphate and KDO carrying side chains of five sugar units, *N*-acetylglucosamine→glucose → galactose→glucose, with a secondary side chain of galactose on the first glucose (Fig. 17).

From our studies (123) as well as those of Nikaido, Stocker et al. (11, 154), and Osborn et al. (167), it was concluded that chemotype Ra, which carries the serological specificity R II, is a galactose, and glucose, which also constitute the R II polysaccharides (91, 93). (ii) Several *Salmonella* O antigens of lower chemotypes cross-react with *R II* antisera (120). (iii) *Salmonella* O antigens originally devoid of R specificity, especially members of the higher chemotypes, cross-react with *Salmonella* R antisera after mild acid hydrolysis, indicating internal R-specific structures (120). (iv) Kauffmann isolated R II mutants from a large number of *Salmonella* S forms belonging to many different serogroups and possessing O antigens of many different chemotypes. The (lipo)polysaccharide antigens of these mutants were very similar or identical serologi-

cally (10). (v) The structure of lipopolysaccharides from R II mutants of the following *Salmonella* species were analyzed: *S. minnesota*, *S. typhimurium*, *S. poona*, and *S. inverness*. Oligosaccharides isolated from partial hydrolysates were identical according to the analytical criteria used (233). (vi) Oligosaccharides recently isolated from partial hydrolysates of O antigens proved to be identical with those isolated from R II polysaccharides (196). (vii) It is obvious that the S-R mutation may be due to the blockage of only one enzymatic step necessary for the biosynthesis of either the core or the specific side chains. It is possible in some cases to obtain the complete parent smooth polysaccharide through bypassing the enzymatic defect by adding the missing sugar to the culture medium (33*a*, 40, 44, 148, 167).

However, the possibility cannot be excluded that in some cases mutants may synthesize polysaccharide structures which do not occur in the wild-type antigen. This may be true for T 1 antigens, which are characterized by a high ribose and galactose content (123*a*).

According to Osborn (164), and in agreement with our own findings (123), the molar ratio of heptose to KDO in R antigens is about 10:1. KDO determinations can be erroneous (*see* 19*a*). As KDO is rather acid-labile, the conditions necessary to liberate KDO from lipopolysaccharides inevitably destroy appreciable amounts. In M antigens, the heptose-glucose ratio is close to 2 (164). On the assumption of a central core of 10 heptose phosphate units, the molecular weight of the M polysaccharide is about 3,700 to 3,800; from the reducing power and the behavior on Sephadex columns, Osborn obtained molecular weights between 4,000 and 5,000 for the M polysaccharide. The structure given in Fig. 17 indicates a molecular weight of about 7,000 to 8,000 as calculated for R II polysaccharides. If an O-specific *Salmonella* polysaccharide with 10 heptose units would contain five specific side chains (because only every second heptose carries one R II-specific side chain starting with glucose) and each side chain would be composed of six repeating pentasaccharide units, then a molecular weight of about 30,000 would be calculated. Various authors have indeed found values of this order for O-specific polysaccharides of the Freeman type (*see* 28).

Although most of the experimental data are in good agreement with the representation shown in Fig. 17, there are still many unsolved problems and the structure might well be oversimplified. Among these problems are the following.

(i) Crucial experiments are still needed to prove that the R II-specific structure is actually the internal backbone of *Salmonella* O antigens. Since it was shown (183) that biosyntheses can be achieved with isolated lipopolysaccharide plus bacterial cofactor (cell wall lipid) as the substrates for sugar transfer, mixed experiments should now be possible: the transfer of glucose, galactose, and acetylglucosamine could be studied with incomplete lipopolysaccharides as acceptor, and with enzymes from different strains in order to determine whether the products of synthesis are the same as in a homologous system. A positive answer would provide strong support for a common basal polysaccharide core in all *Salmonella* O antigens.

(ii) We know very little about the structure of the polyheptose phosphate core, in which presumably also phosphoethanolamine is participating. The linkage of the side chains through glucose was suggested by biosynthetic results. Three heptose-containing oligosaccharides were isolated: one contains additional glucose (151), the second one additional KDO (102*a*), and the third one additional galactose (233), which justifies the small chains drawn in Fig. 17. The biosynthetic relationships between O-specific polysaccharide hapten and the O-antigenic lipopolysaccharide are still to be evaluated.

(iii) We do not know whether the number of repeating units in each side chain is the same, or represents only an average figure.

(iv) The structural identity of all specific side chains in a given O-specific polysaccharide also is not established. In O antigens in which the same immunodominant sugar is bound by different linkages to the same sugar unit of the main chain, as, for example, glucose to galactose in *Salmonella* group B polysaccharides [α-(1 $\rightarrow$ 6) in factor *1* or α-(1 $\rightarrow$ 4) in factor *12*$_2$], the distribution of these secondary side chains along the main chain is not known. The distribution could be random; it could also be that glucose at the end of each chain is linked by -(1 $\rightarrow$ 4)-, whereas the other glucose residues linked to internal galactose constituents are bound -(1 $\rightarrow$ 6)-, or that individual O-specific side chains in the same polysaccharide carry only one or the other specificity (factor *1* chains, factor *12*$_2$ chains). Robbins and Uchida (180) showed that infection of *Salmonella* group E$_1$ organisms with a mutant phage ϵ15b results in the formation of a mutant *Salmonella* E$_2$ antigen which carries two distinct kinds of side chains, one with repeating units of the trisaccharide α-galactose $\rightarrow$ mannose $\rightarrow$ rhamnose, and the other with units of β-galactose $\rightarrow$ mannose $\rightarrow$ rhamnose. We have already discussed the uncertainty of our knowledge of the nature of the terminal nonreducing sugars in the natural polysaccharides. According to methylation data, the O-specific

side chains of the degraded polysaccharide of *S. typhi* would be terminated by different sugars, a result possibly due to the hydrolysis by acetic acid.

(v) The structures of many specific cell wall polysaccharides may not be as simple as those elaborated for groups B and E, as suggested in Fig. 17. This figure is already more complex than that proposed in a preceding review (223). Some labile linkages may exist in the basal core or in the side chains which prevent the isolation of the corresponding oligosaccharides. For instance, it proved difficult to obtain oligosaccharides containing 3/6-dideoxy-hexoses (but see 32*a*). Very small quantities of unidentified oligosaccharides were found [for instance, a disaccharide with mannose and rhamnose in group B (4)] which might be a product of reversion but might also be part of the original structure.

It is difficult also to conceive of the presence of identical chains with simple repeating units in the polysaccharide of group A studied by Tinelli and Staub (239). In this case, it was found that rhamnose and galactose are only partially oxidized by periodate, an observation which seems to indicate that these two sugars are present in the polysaccharide in at least two different linkages.

(vi) Finally, we know practically nothing of the linkage of degraded polysaccharide moieties to each other, to the lipid component, and to protein in the complete antigen.

Biosynthesis of O and R Polysaccharides: S-R Mutations

It was the study of R mutants which gave insight into the biosynthesis of enterobacterial O and R polysaccharides. On the basis of comparative analyses, it was suggested that the lipopolysaccharides of R forms were intermediates in the biosynthesis of the O-specific polysaccharides of wild types (S forms). At present there is no reason to assume that in R cells hexose transfer reactions take place leading to structures which are *not* established in S cells. It became possible, in principle, to reconstruct the general pathway of polysaccharide biosynthesis in *Salmonella* cell walls through the study and serological identification of R II and R I mutants, as well as of M and other mutants. Nikaido, as well as Horecker, Osborn, and associates, were able to reproduce certain biosynthetic steps in vitro, starting from cell wall preparations of mutants containing an incomplete lipopolysaccharide, transferase, and cofactor(s) (*see* 149, 150, 167). Upon addition of the appropriate nucleoside diphospho sugar (UDP-glucose, UDP-galactose), incorporation of the hexose occurred, as shown by isolation of altered lipopolysaccharide. A stepwise transfer of glucose, then galactose, again glucose, and then glucosamine was thus achieved (Fig. 16A). These findings are in agreement with the structural analyses of R II (lipo)polysaccharide as schematized in Fig. 17. They also demonstrate that the biosynthesis of the complex polysaccharide present in R antigens occurs by successive addition of specific sugar residues.

Different mechanisms are involved in the biosynthesis of the O-specific side chains. Incorporation of one sugar is markedly increased by the presence of the nucleotides of the other sugars constituting the chain. Oligosaccharide, and possibly polysaccharide lipid derivatives function as intermediates (Fig. 16B). It seems probable that oligosaccharides (repeating units) are transferred to build up the chains, a mechanism which would favor the formation of main chains, each being terminated by the same sugar, for instance, mannose-abequose in group B.

Blocks in the synthesis of sugars constituting the O-specific main chain, and blocks in any of their transfer reactions, abolish the biosynthesis of repeating units and, consequently, of the chain, thus generally allowing only the synthesis of the R II structure. Many different *Salmonella* genotypes are therefore expressed in one and the same phenotype of R II specificity.

Although most of the R mutants isolated by Kauffmann (10) from *Salmonella* S forms belonged to serotype R II, Stocker and co-workers (11), using other methods of selection, isolated only a few R II strains, but many strains of serotype R I, from the wild type of *S. typhimurium*. Schlosshardt (*see* 123*a*), on the other hand, who isolated R mutants from *S. minnesota* by selection with specific phages, obtained about 20 mutants, none of which belonged to serogroup R II. The cell wall extracts of these mutants belonged to chemotypes Ra, Rb, Rc, and Rd and to another type, Re, with neither hexose nor heptose, but only KDO, ethanolamine, and lipid A. This type represents a mutant with no basal polysaccharide whatsoever. Thus, the methods applied for selecting R strains determine which R mutant will be isolated.

No mutants lacking lipid A have been described. As D-glucosamine is not only a constituent of the R II structure and of the backbone of lipid A (18, 255), but also of the glycopeptide of the rigid cell wall layer [*N*-acetylglucosamine, *N*-acetylmuramic acid (185*a*)], it may be assumed that mutations leading to UDP-*N*-acetylglucosamine deficiency would be lethal. Other mutations, however, as, for instance, defects in transferases or acylases, would probably result in the absence of lipid A.

Kauffmann (93) readily obtained R mutants

from *Salmonella* serogroups with O antigens (lipopolysaccharides) of the higher, more complex chemotypes, but found it very difficult to obtain R mutants from *Salmonella* S forms belonging to chemotype I. Such R forms can be expected to exhibit cross-reactions with the parent O form. In this case, the absence of O specificity cannot be taken as a criterion for purity of the mutant strain.

It is difficult to obtain R forms from *E. coli* strains. A few *E. coli* R strains are well known, like the "classical" *E. coli* K-12, *E. coli* B or Moller's *E. coli* R_1 and R_2 (138). Their genetically related parent strains are not known. Heath and Elbein (33*a*, 65) succeeded in isolating an M form from *E. coli* O111:B4. A complication with *E. coli* S-R mutations is that many strains produce additional K (capsular) antigens; it is not known whether both O *and* K antigens are involved in S-R mutations. It is not known whether the M mutant of *E. coli* O111:B4 isolated by Heath still synthesizes the K (B4) antigen of the parent strain.

It should be mentioned, however, that strains of *S. typhi* (Ty 6 S, Ty 441 Rs) are known which do not possess O antigen (36), though they still contain Vi antigen, which is a capsular antigen like the K antigens of *E. coli*.

From the biochemical and genetic points of view, it is of interest to note that certain enterobacterial strains produce more than one specific polysaccharide. In addition to the O antigen, *S. typhi* produces the Vi antigen. Similarly, numerous *E. coli* strains elaborate additional K antigens, many of which are uronic acid-containing polysaccharides (77, 77*a*, 77*b*, 160*a*). Also, certain *E. coli* species produce various specific polysaccharides (260*a*). For instance, cells of the mucoid type of *E. coli* O59:K(?):H19 synthesize an O antigen (lipopolysaccharide), a K antigen, which is an acid polysaccharide, and M antigen, which is a slime-forming acid polysaccharide probably identical with Goebel's colanic acid (52) (Table 20). This *E. coli* O 59 strain thus produces three polysaccharides of different composition. It would be interesting to study the mechanisms of biosynthesis utilized by these organisms and their genetic implications.

Biological Properties of O and R Polysaccharides and Their Complexes

O and R antigens are situated at the surface of the cell wall (68*b*) and are responsible for the agglutination of the bacteria by corresponding antibodies (193*a*). (Only cells producing Vi or K antigens do not agglutinate with O antisera without previous heating.) One must, therefore, consider the possible influence of cell wall polysaccharide structure on the physicochemical surface properties and on the various biological properties of the cell.

It can be assumed that the capacity to bind water at the cell surface will be significantly influenced by the structure of the polysaccharide. It is well known that highly branched polysaccharides can bind rather large amounts of water. Therefore, the stability and size of the water layer fixed around the bacterial cell surface is certainly influenced by the cell wall polysaccharide and its specific structure. For rough strains, this capacity will be relatively small, and this may be one of the reasons for their well-known tendency toward spontaneous agglutinability.

TABLE 20. *Sugar composition of the lipopolysaccharide, the slime substance, and the acidic polysaccharide isolated from Escherichia coli O59:K(?):H19 mucoid phase, grown at 18 C*

Substance	Sugar composition
Lipopolysaccharide, 2.5% (O antigen)	Glucosamine Heptose Glucose Mannose (trace of galactose)
Slime substance, 9% (M antigen)	Glucuronic acid Galactose Glucose Fucose
Acidic polysaccharide, 2.7% (K antigen)	Galacturonic acid Glucosamine Galactose Mannose

(Jann (77b), see also Ørskov et al. (160a))

Another aspect of biological behavior influenced by the cell wall polysaccharide is related to the relative lipophilic character of the bacterial surface. This can be influenced by the relative proportion of hexoses to deoxy- and dideoxyhexoses. The lyophilic/lipophilic surface character of *Salmonella* strains with O antigens of the lower chemotypes differs significantly from that of the high chemotypes (see Table 5). For the construction of relatively lipophilic cell surfaces by polysaccharides, two principles appear to be utilized by *Enterobacteriaceae*. These could be termed (i) the *deoxy principle* and (ii) the *acyl principle*. The latter is applied by the introduction of *O*-acetyl groups, such as *O*-acetylgalactose of factor *5* in *Salmonella* group B. Bacteria other than *Enterobacteriaceae* can also apply a third principle which could be called (iii) the *O-methyl principle*. Some acid-fast bacteria synthesize *O*-methylated deoxy sugars as constituents of surface

oligosaccharide complexes, but they usually help themselves out with a lot of lipid, too.

These surface properties may be of importance for host-parasite relationships. For instance, rough strains are nonpathogenic and smooth strains of the lower chemotypes are generally less pathogenic than strains of the higher chemotypes; *S. paratyphi C*, however, is an exception. The rough strains are more easily phagocytized and less able to resist the defense mechanisms of the host. The relative lipophilic character of the cellular surface may, therefore, be one, but certainly not the only, determinant of bacterial pathogenicity.

Ørskov et al. (160) found that the fertility rate of *Salmonella* strains, as judged by mating experiments with *E. coli* Hfr strains, was dependent upon the nature of the O-antigenic polysaccharide. The percentage of fertile strains was low (26%) for *Salmonella* strains belonging to groups A to D_1 with O antigens of the high chemotypes (XIV to XVI), but rather high (64%) for *Salmonella* strains with O antigens of the lower chemotypes. This seems to indicate that fertility may also be influenced by polysaccharide-dependent surface properties.

The chemical properties or physicochemical conditions necessary for endotoxicity of *Enterobacteriaceae* and other gram-negative bacteria are unknown. Although no specific hexose constituent of any R or S specific polysaccharide structure can be held responsible for endotoxic properties (123*b*, 145, 260*a*), the question remains as to whether the polysaccharide component of the endotoxic complex contributes anything to these marked biological properties. On the other hand, clear-cut proof is also lacking that the lipid A part of the complex contains structures specifically responsible for endotoxic activities. Ribi et al. (174*a*) have stressed the fact that highly active lipopolysaccharides can be prepared with a very low content of firmly bound lipid, whereas Nakano (145, *see also* 123*a*) has shown that lipopolysaccharides from rough strains with highly incomplete polysaccharide components are almost as toxic as those of the related smooth parent strain. Therefore, it would seem necessary to focus attention on the chemically simplest endotoxic complex to determine the minimal chemical requirements for endotoxic activities.

Immunological Properties of O Polysaccharides and O Factors—Conversion by Phages

Studies on R antigens supplied confirmation that different R specificities exist, as suggested earlier by Kauffmann (88). However, most of the immunological studies thus far have been carried out with O factors which characterize the serotypes in the Kauffmann-White scheme. These are oligosaccharides composed of two to four (or more) sugar units present on the long side chains. They represent, in part or completely, the determinant group of these chains. The sugar component of O factors best adapted to the specific sites of the antibodies (the best inhibitor of the factor-antifactor reaction) is not necessarily a nonreducing terminal sugar. It may be one of the internal units of the long side chains (for instance, α-acetylgalactose of factor 5, see Fig. 18). We have proposed to call these sugars (either nonreducing terminal or internal) *immunodominant*.

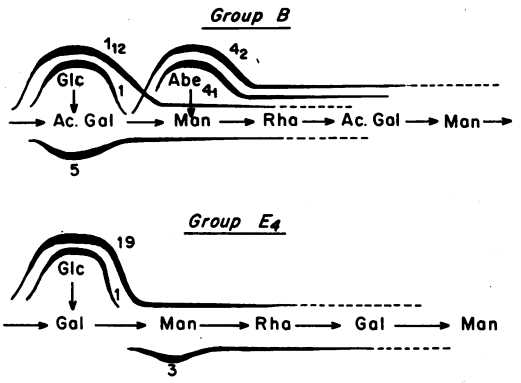


FIG. 18. *Factors 1, 1_{12}, 5, 4_1, and 4_2 on a repeating unit of group B polysaccharide. Factors 1, 3, 19 on a repeating unit of group E_4 polysaccharide.*

Distinct factors of a given O antigen may possess different immunodominant sugars (*5*, *1*, and *4*) or the same immunodominant sugar (*1* and 1_{12}; 4_1 and 4_2 of Fig. 18). In this latter instance, the factors differ by the length of the oligosaccharide which carries their specificity.

Factors determined by cross-agglutination of bacteria in rabbit antisera are never shorter than a disaccharide. This means that up to now no specific sites of rabbit antibodies have been found corresponding only to the immunodominant sugar. In contrast, such antibody reactive sites have been found in other species (horse, goat, hen), and cross-reactions have been observed between factors which have only the same immunodominant sugar in common and which do not cross-react in rabbit antisera.

Another difference exists between antibodies produced by horses and rabbits. For instance, in group E_4 polysaccharides (factors *1*, *3*, *19*) containing the repeating unit shown in Fig. 18, it was possible by specific inhibition to determine four immunodominant sugars, glucose, galactose, mannose, and rhamnose. It appears that each constituent of a chain can play the role of an

immunodominant sugar, as has also been found with pneumococcal polysaccharides (66*a*). However, studies with rabbit sera revealed only two immunodominant sugars: glucose for factors *1* and *19*, mannose for factor *3*. The basis for the differences between sites of antibodies obtained from various animal species is still unknown.

Five of the factors (*1*, 1_{12}, 4_1, 4_2, *5*, 12_1, 12_2) characterizing *S. paratyphi* B are given in Fig. 18, which represents one repeating unit of the specific polysaccharide extracted from this organism. The structure of 12_1 is not known. Factor 12_2, containing a terminal glucose, like factors *1* and 1_{12} but differently linked, cannot be present on the same unit. It can be seen that the chemical structure of a single O factor may include parts of the structure of other O factors. Factors may often overlap structurally.

The structural relationships between factors *1* and 1_{12}, and 4_1 and 4_2, have been determined, but those between factors 1_{12}, and 4_1 (or 4_2) have not yet been studied. However, factor 1_{12} was found *not* to be identical in *Salmonella* groups B and D_1, probably because the closely attached factors *4* (in group B) and *9* (in group D_1) possess different dideoxy-hexoses.

The mechanism of the formation of antibodies related to oligosaccharides, such as factors *4*, *5*, and 12_2, which are carried by the same oligosaccharide unit, is not known. Do they reach the antibody-synthesizing cell as such, or do they reach these sites in the form of different oligosaccharides after cleavage by different enzymes?

Advances in our knowledge of the chemical structure of O factors have been very useful in providing a means of approaching the puzzling problems posed by the conversion of *Salmonella* by phages.

Changes of O specificity following the action of lysogenic phages, originally discovered by Japanese workers, could be related to uniquely limited changes in the structure of the specific polysaccharide (*see* 225*a*), e.g., appearance of a new secondary side chain of glucose, the change from an α to β linkage, or the change from a $1 \rightarrow 4$ to a $1 \rightarrow 6$ linkage. It was thus established that after phage conversion, as after S-R mutation, a profound change of specificity (appearance of a new factor, two new factors, or even three factors) might be related to an apparently single change in the enzymatic equipment of the bacteria. Phage conversion may be related to the appearance or derepression of a single enzymatic system. Whether the genetic information on which this new enzymatic system depends belongs to the genome of the phage (appearance of a new enzyme) or to the bacteria (derepression of an enzyme) is not known. However, the new chemical constitution observed after phage conversion can also be found in nonlysogenic *Salmonella*. Factor *1* exists in group E_4, in which no prophage has yet been demonstrated; mannose-(1→6)-galactose present in bacteria of group B after conversion by phage 27, is present in all bacteria of group E; factor *34* of E_3 is due to glucose-$(1 \rightarrow 4)$-galactose, like factor 12_2 of groups A, B, and D_1 which does not seem related to any phage.

According to these data, the potential presence in the bacteria of the new enzymatic system would seem reasonable, the role of the phage being limited only to its expression. Preliminary chemical data seem to be in agreement with this assumption (4).

Approaches to a Genetic Classification of Salmonella

In the present status of knowledge of bacterial genetics, a valid system of bacterial classification should be constructed upon genetic criteria (*see* 181, 188). Since the specificity of O factors depends upon their chemical constitution, which is in turn due to the activity of enzymatic systems controlled by the genetic apparatus, the Kauffmann-White scheme based on these factors can be considered as a first attempt toward a genetic classification (89). Actually, a relationship between groups A, B, and D_1 emerges from their antigenic O formulas. They possess almost identical factors 1_{12} and identical (or almost identical) factors 12_2. The relationship among the groups is also reflected in their behavior towards phage. All are sensitive to phages P22 and 27, and a common factor appears in the three groups after conversion by these phages.

Two recent findings have brought forth new evidence for the close genetic relationships among groups A, B, and D_1. (i) Matsuhashi and Strominger (129) found that CDP-paratose is the precursor of CDP-tyvelose, a specific 2-epimerase catalyzing the interconversion of the two 3,6-dideoxy-hexose nucleotides (enzyme 7 in Fig. 10; see also Table 1). Paratose is specific for O antigens of *Salmonella* group A and tyvelose is specific for group D_1. Serotypes of group A may therefore represent CDP-paratose epimeraseless mutants of *Salmonella* group D_1 strains. This would explain the close relationship of the two groups, as stressed by Kauffmann (86) on the basis of bacteriological findings. (ii) Mäkelä (127) showed that the presence of abequose in group B and of tyvelose in group D_1 is genetically controlled by two alleles present on the same locus of the genome.

Chemical results agree very well with such a relationship. Structural analyses of *S. typhi* (group D_1) and *S. paratyphi* B (group B) have

shown that the main side chains of both serotypes consist of the same sequence of galactose→ mannose → rhamnose on which only the secondary side chains of 3,6-dideoxyhexose differ. The nature of the linkage in the disaccharide galactose→mannose has been established only in *S. bredeney* (group B), but the similarities between the chromatograms of the hydrolysates of the three groups (4) as well as between the factors

1$_{12}$ galactose-(1→6)-galactose→mannose → rhamnose

and

12$_2$ galactose-(1→4)-galactose→mannose → rhamnose

strongly suggest that the linkage is the same in groups D_1 and A.

Thus, it appears that the Kauffmann-White scheme is helpful for a genetic classification. However, its primary goal was a practical, diagnostic one, and cross-reactions have been limited to those which are most useful for this purpose. As previously emphasized, the scheme has been deliberately simplified. For instance, such serogroups as D_2 (*9, 46*) and E_1 (*3, 10*), which do not seem related according to their formulas, actually share a common factor, as shown by Kauffmann (87, 90). This was recently confirmed by Le Minor (109), who also found that *Salmonella* of group D_2 were sensitive to phages ε15 and ε34, resembling *Salmonella* of group E_1 and in contrast to *Salmonella* of group D_1. The converted D_2 bacteria possess factors *15* and *34* similar to those converted from group E. It would thus seem that serogroup D_2 is intermediate between groups D and E. Moreover, after their conversion by phage 27, *Salmonella* of groups A, B, and D_1 acquire a new common factor (*27*) and then cross-react with *Salmonella* of group E, similarly to *Salmonella* of group D_2 (Le Minor, Staub, *unpublished data*). These immunological results can be seen in the scheme shown in Fig. 19. It would thus appear that groups D_1 and E are more closely related genetically than is apparent from the Kauffmann-White scheme.

Chemical investigations on group E polysaccharides have shown that all the subgroups possess a main side chain containing the same sugars in the same sequence as groups B and D_1, but with different linkages. Groups D_1 and E therefore appear more alike chemically than serologically. It was also shown that after conversion by phage 27 the disaccharide galactose → mannose of group B (and probably of group D_1) acquires the same linkage as the disaccharide galactose→ mannose of group E (see Table 21) (4).

Since *Salmonella* D_127^+ and D_2 seem to represent intermediates between bacteria of groups D_1 and E, a tempting working hypothesis would be that in *Salmonella* of group D_2 the polysaccharide possesses the same linkage of galactose → mannose as that in *Salmonella* D_127^+ and in group E. This hypothesis is being tested.

Similarly, groups C_4 and H, which both possess factors *6* and *14*, are certainly closely related. *Salmonella* strains of group K (*18*) do not show any immunological relationship with the former groups, except *S. siegburg* which possesses factors *6*, *14*, and *18*. Recently Le Minor found (110) that factors *6* and *14* of *S. siegburg* are correlated with the presence of a prophage, and that they can be acquired by other serotypes of group K. It is thus very probable that groups C_4, H, and K are genetically related, similarly to groups A, B, D_1, D_2, and E.

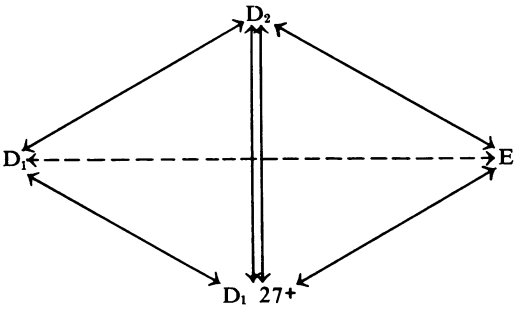


FIG. 19. *Serological relationships among Salmonella strains of groups* D_1, D_1 27^+, D_2, *and E. Broken line indicates no cross-reaction; solid line, cross-reaction. Between* D_2 *and* D_1 27^+, *two cross-reactions can be demonstrated: sera anti-*D_2 *absorbed by* D_1 *still cross-react with* D_1 27^+.

Contrary to these findings, the chemical constitution of the specific side chains of some serogroups such as G, N, and U (see Table 8) are quite distinct, and it seems highly probable that these groups are genetically very remote from each other and the aforementioned groups.

To summarize, it seems that the knowledge of the chemical constitution of the side chains would permit a rearrangement of the serogroups of *Salmonella* unrelated to the Kauffmann-White scheme; therefore, a systematic investigation of these constitutions would be desirable to establish a genetic classification of *Salmonella*. A first attempt to classify these organisms chemically has been made in terms of their chemotypes. It can be seen from Tables 5 and 6 that in the genera of *Escherichia* and *Salmonella* many organisms that do not cross-react serologically may belong to the same chemotype. For instance, the (lipo)polysaccharides of twelve O groups of *E. coli* belong to chemotype I, 9 to chemotype II, and 16 to chemo-

type VII. In addition, five *Salmonella* O groups belong to chemotype I, four to chemotype II, and two to chemotype VII. Among these *Salmonella* and *E. coli* organisms, only two partially cross-reacting pairs have been detected, one belonging to chemotype II and another to chemotype VII. In other words, many serologically different enterobacterial strains synthesize and incorporate

(i) A common polysaccharide core constituted of galactose, glucose, glucosamine, heptose, and KDO exists in all *Salmonella*. The two last sugars have not yet been found in the side chains, but three others may or may not be present in the chains. For example (Table 8), although the O antigens of *Salmonella* groups G, N, and U belong to the same chemotype, VI, their specific side

TABLE 21. *Intergroup relationships among Salmonella groups A, B, and E**

Serogroup	O Factors	Structure of the repeating unit
E_1	3, 10	→ α-acetylgalactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
E_2	3, 15	→ β-galactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
		α-glucose (1 → 4)
E_3	(3), (15), 34	→ β-galactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
		α-glucose (1 → 6)
E_4	1, 3, 19	→ α-galactose-(1 → 6)-α-mannose-(1 → 4)-rhamnose →
		α-tyvelose
D_2	3, (9), 46	→ galactose-(1 → 6)-mannose-(1 → x)-rhamnose → (hypothetical)
		α-abequose (1 → 3)
B	4_1, 12_1, 27, 27B	→ galactose-(1 → 6)-β-mannose-(1 → 4)-rhamnose →
		α-abequose (1 → 3)
	$4_1, 4_2, 12_1$	→ galactose-(1 → 4)-β-mannose-(1 → 4)-rhamnose →
		α-glucose (1 → 4); α-abequose (1 → 3)
	$4_1, 4_2$, 5, 12_1, 12_2	→ α-acetylgalactose-(1 → 4)-β-mannose-(1 → 4)-rhamnose
		α-tyvelose
D_1	$9_1, 12_1, 12_3$	→ galactose-(1 → 4)-mannose-(1 → x)-rhamnose →
		α-glucose (1 → 4); α-tyvelose
	$9, 12_1, 12_2, 12_3$	→ α-galactose-(1 → 4)-mannose-(1 → x)-rhamnose →

* Results in group B were obtained with *S. bredeney*. The disaccharide mannosyl-rhamnose obtained from group B polysaccharides is resistant to the action of α-mannosidase (4) which splits the corresponding disaccharide obtained from group E polysaccharides (4, 179). The 1 → 4 linkage in group D_1 oligosaccharides is suggested by immunological and chromatographic similarities between groups B and D_1, as well as by their sensitivity toward the same phages.

the same combination of sugars into their cell wall lipopolysaccharides. Most of these lipopolysaccharides must be structurally more or less differentiated as expressed by the many serological O specificities. However, some facts argue against the biological significance of the classification into chemotypes.

chains contain different sugars. In group N, the chains are composed of galactosamine, glucose, and fucose; in group G, additional galactose and in group U additional galactose and glucosamine are present (196).

(ii) Short secondary side chains appear or disappear on a serotype as a consequence of form

variation (e.g., glucose of factor 12_2) (94), mutation (acetyl of factor *5*), or phage conversion (glucose of factor *1* and *34*).

It is therefore apparent that a knowledge of the nature of the sugars does not provide a sufficient basis for a valid biological classification. The exact structural composition of the side chains must be known to establish such a classification of *Salmonella* and *E. coli*. When the total number of known serogroups is considered, the accomplishment of this goal might appear to be rather remote. However, new serological relationships might be observed; new and simpler mutants, such as the 12_2^- of groups A, B, D_1, might be found; and new conversions by phages might be obtained. Such additional information would greatly assist immunochemists in choosing the appropriate serogroups for more economic investigations.

A final and important question is whether, in the family of *Enterobacteriaceae*, genera other than *Salmonella* manufacture the same or a similar basal core polysaccharide. Beside L-glycero-D-mannoheptose in *Salmonella*, various other stereoisomeric heptoses have occasionally been found in gram-negative bacteria (see Table 3). Recently, two different heptoses were found to be present in the lipopolysaccharide of a *Proteus mirabilis* strain: L-glycero-D-manno-heptose, which could be isolated as a phosphate, and D-glycero-D-mannoheptose (3*a*, see also 1*a*).

Secondly, in the genus *Escherichia*, a few strains were detected (*E. coli* O17, O44, O59, and O77) which do not contain galactose in their O-specific polysaccharide (see *E. coli* chemotypes XVII and XIX in Table 6). One may assume that R forms of these strains do not produce galactose-containing R polysaccharides, and therefore do not use galactose as a constituent of their basal core polysaccharide. The same would hold for *Shigella dysenteriae* with respect to glucose. The O-specific polysaccharide of the wild type, analyzed by Morgan (139) and later reinvestigated by Davies et al. (28), does not contain glucose. We have begun to repeat the analyses of galactose-free *E. coli* polysaccharides, especially with regard to the isolation and analysis of R mutants of the wild strains (*see* 24). Further comparative analyses of the core polysaccharide present in R mutants will certainly yield more insight into intergenetic relationships between *Enterobacteriaceae*.

In spite of the many unsolved problems, both old and new, it is clear that the immunochemical investigations of *Salmonella* and related organisms carried on in different laboratories during the past decade have done more than "provide information on the apparent mosaic structure of antigens," as predicted by Landsteiner. They have also brought new insight into the genetic relationships among strains belonging to this genus of *Enterobacteriaceae*, and into the biochemical and genetic background of their antigenic modifications.

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